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Survival of Bifidobacterium in milk and during simulated gastrointestinal tract transit

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**Survival of *Bifidobacterium* in milk and during simulated
gastrointestinal tract transit**

by

Ean-Chee Ng

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Major: Food Science and Technology

Program of Study Committee:
Terri D. Boylston, Major Professor
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Ames, Iowa

2006

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This is to certify that the master's thesis of

Ean-Chee Ng

has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy

This thesis is dedicated with love to my parents, Swee Keong and Soo Lan, who have made all my achievements possible and to the memory of my beloved late brother, En-Sean..

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ABSTRACT

The nutritional value of milk can be improved through the incorporation of *Bifidobacterium*. The objective of the study was to evaluate the viability of *Bifidobacterium* in milk and simulated gastrointestinal tract transit and lactose utilization during refrigerated storage. Three experiments were conducted. *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bifidobacterium breve* and *Bifidobacterium bifidum* were inoculated into milk at 10^8 CFU/mL and viability in milk and simulated gastrointestinal tract transit and lactose utilization were studied in the first experiment. In the second experiment, *B. bifidum* was inoculated at 10^4 , 10^5 , and 10^6 CFU/mL to determine the effect of inoculation level. Finally, milk inoculated with 10^6 CFU/mL *B. bifidum* was supplemented with 2% and 3% (w/v) whey protein concentrate (WPC) or fructooligosaccharides (FOS) to determine the effect of supplements. Samples were stored at 4°C for 15 days for all experiments. Microbial viability in milk and lactose content were determined at 3-day intervals. Viability after 4 hours treatment with simulated gastric juice (pH 3.0) and 1% (w/v) bile solutions at 37°C were determined on days 0, 6, 12 and 15. Samples were plated on MRSC agar and incubated anaerobically. Lactose content was determined using an enzyme assay in the first experiment.

Survivability in milk over storage period was not significantly different among each strain ($p < 0.05$). *B. bifidum* had a significantly lower survival rate after treatment with bile solutions at day 15 ($p < 0.05$). However, microbial counts exceeded 10^6 CFU/ml for all strains during storage and after simulated gastrointestinal transit. Only *B. bifidum* hydrolyzed lactose, and, at day 15, 31% lactose hydrolysis was observed. No significant

changes in viability of *B. bifidum* inoculated at 10^4 – 10^6 CFU/mL occurred during storage and simulated gastrointestinal transit. Viability of *B. bifidum* was not affected significantly by WPC supplement. FOS affected the viability of *B. bifidum* in milk with significantly lower counts on day 12 and 15 and a better survival after treatment with bile solutions at day 15 for samples supplemented with 2% FOS ($p < 0.05$).

INTRODUCTION

Bifidobacterium are widely used as probiotic microorganisms to develop functional foods. *Bifidobacterium* are gram positive, anaerobic microorganisms that are normal inhabitants in the human colon. In the past few years, *Bifidobacterium* have been associated with several health benefits including the inhibition of pathogenic bacteria such as *Escherichia. coli* O157:H7, improvement of lactose tolerance in lactose malabsorption individuals, prevention or treatment of diarrhea, decreased incidence of childhood atopic diseases, reduction of serum cholesterol levels and, possibly, prevention of colon cancer (Brady and others 2000; Marteau and Boutron-Ruault 2002; Ouwehand and others 2002; Lin 2003; Bergonzelli and others 2005; Sullivan and Nord 2005). Over the past few years, with the increased demand for healthy foods beyond their nutritional value and the health benefits claimed by *Bifidobacterium*, functional foods with the incorporation of viable *Bifidobacterium* have been extensively studied and developed (Scheinbach 1998).

To obtain the claimed health benefits, the functional food must contain viable *Bifidobacterium* with at least 10^7 CFU/g or mL of the product, which is a challenge because *Bifidobacterium* are anaerobic microorganisms and are relatively sensitive to a low pH environment (Ross and others 2005). Therefore, not all food systems are a suitable environment for the survival of *Bifidobacterium* and challenges have to be overcome to produce functional foods that maintain a sufficient amount of viable *Bifidobacterium* throughout the shelf life of the food (Saarela and others 2000; Biavati and others 2000). For this reason, an intensive research effort has focused on protecting the viability of the *Bifidobacterium* during product manufacture and storage. Studies with yogurt and fermented

milk have demonstrated that the viability of the *Bifidobacterium* can be enhanced by supplementing those products with whey protein concentrate or fructooligosaccharides during the fermentation process (Gibson and Wang 1993; Bruno and others 2002; Martín-Diana and others 2003; Akalin and others 2004; Janer and others 2004).

In addition to the stability of *Bifidobacterium* throughout the shelf life of the food, survivability of the organism during gastrointestinal tract transit is another challenge. In order to deliver the beneficial effects, *Bifidobacterium* have to survive through the gastrointestinal tract transit in sufficient amounts to be effective. Gastric juice in the stomach and the action of bile salts in the intestine are harsh conditions for the survivability of the *Bifidobacterium* (Bezkorovainy 2001). Acidity in gastric juice and the antimicrobial action of the bile salts cause damage to the cells and thus decrease their viability.

Several different approaches have been taken to increase the survivability of *Bifidobacterium* during gastrointestinal tract transit. Microencapsulation of *Bifidobacterium* cells in carrier materials, such as cellulose acetate phthalate, gelatin, soluble starch, skim milk and gum arabic, resulted in increased viability when treated with simulated pH 2.0 gastric juice and 2.0% (w/v) bile salts in comparison to the free cells (Rao and others 1989; Lian and others 2003). Immobilization of *Bifidobacterium* cells in calcium alginate beads and gellan-xanthan beads showed a significantly better survival when treated with simulated gastric juice as well (Lee and Heo 2000; Sun and Griffiths 2000). Although these novel technologies seem promising, the production cost may be ineffective. The more economical way to deliver *Bifidobacterium* to the consumer is to develop *Bifidobacterium* supplemented food. Studies has shown that *Bifidobacterium* in fermented milk have increased survivability

in simulated gastric juice treatment and *in vivo* gastrointestinal tract transit (Berrada and others 1991; Pochart and others 1992).

Incorporation of *Bifidobacterium* into dairy products has been extensively studied. Cheese, yogurt, fermented milk and fresh milk with *Bifidobacterium* are widely available in the market. Milk is an excellent carrier medium for the delivery of *Bifidobacterium* into the human gut. Milk proteins function as buffering agents and protease inhibitors and thus help to protect the *Bifidobacterium* during the upper gastrointestinal tract transit (Charteris and others 1998). Moreover, the addition of milk protein to the gastric juice will increase the pH of the gastric juice and, hence, provide a more favorable environment for the survivability of the *Bifidobacterium*. In addition, adhesions of the microorganisms to human intestinal cells are critical in order to permanently establish *Bifidobacterium* in the human gut. Survivability and adhesion of the probiotic bacteria to human intestinal cells are improved with milk as the carrier (Conway and others 1987).

The overall objective of our study was to examine the viability of *Bifidobacterium* spp. inoculated into milk over a 15-day storage period and their survivability after simulated gastrointestinal tract transit. In this study, only *Bifidobacterium* species found in human were used. In order for humans to obtain the health benefits of *Bifidobacterium*, it is recommended, based on scientific beliefs, that human strains are consumed (Saarela and others 2000).

In the first study, the survivability and lactose utilization rate of *B. infantis* R0033, *B. longum* R0175, *B. breve* R0070 and *B. bifidum* R0071, inoculated at $8 \log_{10}$ CFU/mL, were evaluated. In the second study, the *Bifidobacterium* strain with the highest lactose utilization rate was selected and inoculated at lower concentrations, 4, 5, and $6 \log_{10}$ CFU/mL. The effects of the inoculation levels on their survivability over the storage period and during

simulated gastrointestinal tract transit were evaluated. Finally, whey protein concentrate and fructooligosaccharides supplements were added to the milk and the effects of the supplements on the survivability of *B. bifidum* during the storage study and after the simulated gastrointestinal tract transit were evaluated.

The hypothesis of this study is that *Bifidobacterium* strains, inoculation levels and the addition of supplements will affect the viability of the *Bifidobacterium* in milk and simulated gastrointestinal tract transit during a 15-day storage period, and *Bifidobacterium* will utilize the lactose in the milk.

LITERATURE REVIEW

Probiotic Bacteria

The consumption of fermented dairy products has a long history that dates back to many centuries ago even before the discovery of microorganisms (Fuller 1992). It was one of the oldest methods used to preserve and produce foods (Leahy and others 2005). In pre-biblical times, humans consumed fermented products and the health claim stated in the Persian version of the Old Testament (Genesis 18:8) was that “Abraham owed his longevity to the consumption of sour milk.” In 76 BC, fermented milk products were used therapeutically by Roman historian, Plinius, to treat gastrointestinal diseases (Schrezenmeir and de Vrese 2001). However, the concept of probiotics did not emerge until the early 1900s when Eli Metchnikoff in Pasteur Institute in Paris discovered the benefits of lactobacilli. Metchnikoff (1907), the Russian born Nobel Prize recipient, recognized the positive roles of probiotics and suggested that “The dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and replace the harmful microbes by useful microbes”. In addition to Metchnikoff’s finding, Henry Tissier, a French pediatrician, discovered lower counts or the absence of Y-shaped bacteria in infants with diarrhea compared to healthy infants. Tissier then proposed that the consumption of these “bifid” Y-shaped bacteria could help to restore the healthy gut flora and possibly treat diarrhea. Metchnikoff and Tissier’s findings and publications in early 1900s increased the scientific interest in probiotics (Rašić and Kurmann 1983). However, the results were not always positive and the findings were subjective. Thus, the probiotic concept was regarded as not scientifically proven.

In the past two decades, the improvement in the identification and isolation of probiotic cultures has consequently helped to provide evidence that support the health claims related to the consumption of probiotics. The increased interest in consuming probiotics has drawn the attention of the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United Nations and resulted in the definition of the term probiotic. Probiotic was originally derived from the Greek language and means 'for life'. In 1965, Lilly and Stillwell was the first to introduce the term probiotic as "substances secreted by one microorganism which stimulate the growth of another". The term probiotic has been redefined by many other researchers throughout the years, including Parker (1974), the first researcher who used the term probiotic in the sense that it is used today. In 2001, in an Expert Consultation meeting organized by WHO/FAO, the definition of probiotic was proposed as "live micro-organisms which when administered in adequate amounts confer a health benefit on the host". This definition was widely used and in 2002, Marteau and others redefined probiotic as "microbial cell preparations or components of microbial cells that have a beneficial effect on health and well-being". Even though the definitions of probiotic may vary, the benefits of probiotic microorganisms are normally linked to the improvement of human health.

Lactic acid producing bacteria, *Lactobacillus* and *Bifidobacterium*, are the most common probiotic bacteria that are incorporated into food for therapeutic purposes (Roberfroid 2000b). Incorporation of probiotics into food is well accepted by the consumer due to their positive association with maintaining a healthy gastrointestinal tract and their 'generally recognized as safe' (GRAS) status under the Food and Drug Administration (FDA) regulation (Svensson 1999; Sullivan and Nord 2005).

Health Benefits of Probiotic Bacteria

Numerous clinical studies have identified several different health benefits associated with the incorporation of probiotic bacteria into the diet. Prevention of diarrhea, treatment of inflammatory bowel diseases, alleviation of the symptoms of lactose intolerance, decreased incidence of atopic diseases, possible reduction in the risk of colon cancer, and reduction of serum cholesterol have been linked to the consumption of probiotic bacteria (Scheinbach 1998; Lin 2003; Servin 2004).

Diarrhea

Diarrhea has been associated with the disruption of the balance of normal gut microflora. Gut microflora possess a quality known as colonization resistance that could help to prevent the overgrowth of pathogens in the gut. The disruption of the equilibrium of the gut microflora could lead to the overgrowth of pathogenic microorganisms, and the toxins produced by these pathogens can cause diarrhea (De Roos and Katan 2000). Probiotic bacteria are believed to aid in colonization resistance and restore the balance of the gut microflora. Studies have been conducted on the effectiveness of probiotic bacteria on minimizing or preventing the risk of infectious diarrhea, traveler's diarrhea, acute rotavirus diarrhea and antibiotic-associated diarrhea (Bergonzelli and others 2005; Sullivan and Nord 2005).

Infectious Diarrhea

The use of probiotic bacteria as prevention or treatment of infectious diarrhea has been suggested by several research studies. In an *in vitro* study, Gibson and Wang (1994) incubated *Bifidobacterium infantis* with pathogenic microorganisms, *Escherichia coli* and

Clostridium perfringens in various fermentation systems. The growth of the pathogens declined by approximately 0.4 and 0.5 log₁₀ CFU/mL, respectively, even though the pH of the growth media was maintained at neutral. In an *in vivo* study in laboratory animals, Silva and others (2004) found that when mice received 10⁸ CFU/mL *Bifidobacterium longum* supplemented in milk in their diet, they had a 40% higher chance of surviving than the control mice when *Salmonella typhimurium* (10¹ CFU/mL) suspension was fed. Asahara and others (2004) showed that mice fed with 10⁸ CFU/mL *B. breve* had a higher survivability rate compared with control mice when Shiga toxin-producing *Escherichia coli* O157:H7 (10³ CFU/mL) was fed. Besides inhibiting these pathogens, results from several *in vitro* and *in vivo* studies have revealed that *Bifidobacterium* species are effective in inhibiting the pathogenic effect or the growth of other pathogens including *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium, *Salmonella enteritidis* ssp. Typhimurium, *Clostridium perfringens* and *Escherichia coli* O157:H7 (Silva and others 1999; Asahara and others 2001; Touré and others 2003; Gagnon and others 2004). Results from these studies suggested that with the consumption of probiotic bacteria, the risk of developing diarrhea caused by foodborne pathogens could be minimized.

Traveler's Diarrhea

Traveler's diarrhea is one of the most frequently encountered health problems among tourists, especially tourists travelling to underdeveloped countries. Tourists travelling from a developed country to a developing country have a 20–50% risk of suffering from traveler's diarrhea (Ericsson 2003). The most common causes of traveler's diarrhea are the enterotoxin-producing *Escherichia coli*, followed by *Campylobacter jejuni*, *Salmonella typhimurium*, *Shigella* species and other harmful bacteria and viruses (Dupont and Ericsson

1993; Casburn-Jones and Farthing 2004). A study conducted by Hilton and others (1997) with *Lactobacillus casei rhamnosus* GG as the probiotic supplement showed that the incidence of diarrhea was lower in the supplemented group. Oksanen and others (1990) conducted a placebo-controlled, double-blinded study on subjects traveling to two different destinations in Turkey. A 5.5% lower incidence of diarrhea in the probiotic group, with the *L. casei rhamnosus* GG supplement, compared to the placebo group were observed in tourists traveling to Alanya. However, the same effect was not observed in subjects who traveled to another destination, Marmaris, when a similar study was conducted. Even though these results showed that probiotics helped in preventing traveler's diarrhea, the methodology used was insufficient and some of the results are not significant. Thus, the evidence for the beneficial effects remains unconvincing and no clear conclusion could be drawn (DuPont 1997).

Acute Rotavirus Diarrhea

Acute rotavirus diarrhea is one of the major causes of diarrhea in infants and young children and accounts for approximately 45% of all diarrhea in children worldwide (Sullivan 2005). Each year, nearly 500,000 children die due to severe rotavirus disease and it is estimated that, by age 5, almost every child will have rotavirus diarrhea (Parashar and others 2003). WHO (1995) recommended the use of oral rehydration salts (ORS) to replace the fluid and electrolyte losses in treating this disease. Efforts have been made to develop a vaccine for this disease; however, before an effective vaccine is available, some preventive actions can be taken to reduce the incidence of rotavirus diarrhea in children (Rolfe 2000).

Research from several large and well-designed clinical trials carried out in Finland, Pakistan, Europe, Peru and the United States have shown the effectiveness of probiotics

supplements in the treatment or prevention of rotavirus diarrhea in children. Guandalini and others (2000) added 10^{10} CFU/250mL *Lactobacillus* GG to the ORS for children with acute-onset diarrhea. The diarrhea lasted longer than 7 days for 2.7% of the children in the probiotic groups compared to 10.7% of the children in the placebo group. Other studies also showed that when probiotic bacteria were either given as a supplement or in fermented milk, the risk of rotavirus diarrhea decreased in healthy children and the duration of diarrhea was significantly shortened in the children infected with rotavirus diarrhea (Isolauri and others 1991; Saavedra and others 1994; Raza and others 1995; Guarino and others 1997; Shornikova and others 1997; Oberhelman and others 1999).

Antibiotic–Associated Diarrhea

Antibiotic–associated diarrhea (AAD) is defined as diarrhea that develops between a few hours after the initiation of the antibiotic treatment to six to eight weeks after the end of the treatment (Beaugerie and Petit 2004). AAD occurs in 5 to 25% of the patients receiving antibiotic therapy, although the reason behind this is not fully understood (Bergogne-Bérézin 2000). Surawicz (2003) suggested that the disruption of the normal gut microflora has increased the risk of infection with pathogens, such as *Clostridium difficile*. *C. difficile*, the most common cause of AAD, accounts for 10–20% of AAD in antibiotic therapy patients. In addition, 20% of the hospital patients may be infected with *C. difficile* (Bartlett 2002; Plummer and others 2004).

C. difficile colonizes and grows in the human colon and the toxins produced could lead to colonic damage. Antibiotic treatment is normally used to treat this infection but the recurrence of the infection after the therapy is very common (Surawicz 2003). Barbut and others (2000) estimated that within 2 months after the first episode of *C. difficile* infection,

patients will have 15–35% risk of re-infection. The use of probiotics is recommended to restore the normal gut microflora to prevent the re-infection. Plummer and others (2004) studied patients receiving antibiotic therapy with both *Bifidobacterium* and *Lactobacillus* or placebo as supplements. Lower risks of developing diarrhea were observed in the group receiving both *Bifidobacterium* and *Lactobacillus* as supplements compared to the control group who received a placebo as a supplement. In addition to this study, additional research with different strains of probiotics, such as *Bifidobacterium longum*, *Lactobacillus* GG and *L. casei* GG showed a significant effect on the prevention and treatment of AAD (Colombel and others 1987; Siitonen and others 1990; Young and others 1998).

The mechanisms of how probiotic bacteria function on the prevention of diarrhea are not fully understood. The production of acid, hydrogen peroxide or antimicrobial agents, and the competition for adhesion receptors and nutrients might play an important role (Servin 2004).

Inflammatory bowel diseases

Inflammatory bowel diseases (IBD) are referred as ulcerative colitis, Crohn's disease and pouchitis (Lin 2003). Genetic susceptibility, environmental factors and immune dysregulations are the underlying causes of IBD. Even though these are the main factors that cause IBD, the colonization of pathogenic bacteria is required to express the disease fully (Shanahan 2002). Currently, antibiotic treatment is the commonly applied treatment for IBD patients. However, antibiotic treatment is not encouraged for the treatment of uncomplicated IBD patients (Feagen 1997) and chronic use of antibiotic treatment can lead to toxicity, side effects, risks of bacterial resistance and overgrowth of bacteria in the patients (Shanahan

2004). Therefore, the alteration of the gut microflora is a more promising way to treat IBD patients.

Studies using a non-pathogenic strain of *Escherichia coli* have shown to be as effective as mesalazine in treating ulcerative colitis patients, these results have further increased the interest in the use of non-pathogenic bacteria to replace antibiotic treatment in IBD patients (Kruis and others 1997; Rembacken and others 1999). Investigators in several animal model studies and human trials demonstrated the possible use of probiotics in treating IBD. O'Mahony and others (2001) studied interleukin-10-deficient mice with *Lactobacillus salivarius* supplemented in their diet compared with control non-probiotic fed mice. A lower histological index score on the ileum, caecum and colon tissues, based on degree of inflammation, was scored by the probiotic fed mice compared with the control mice.

Gionchietti and others (2000, 2003) developed a new probiotic therapy, VSL#3, to treat the onset of acute pouchitis and chronic pouchitis. VSL#3 is a highly concentrated probiotic therapy with eight different strains of lyophilized probiotic bacteria, which consisted of 4 strains of *Lactobacillus*, 3 strains of *Bifidobacterium* and 1 strain of *Streptococcus* at 3×10^{11} cells per gram of live microorganisms. A cocktail of probiotic bacteria is recommended compared with the use of single strains of probiotic bacteria because one strain of probiotic bacteria might work on certain patients or on a certain phase of the disease in a patient but not others (Bengmark 1996). Results of a double-blinded, placebo-controlled trial was conducted and the results showed that treatment with VSL#3 were effective in preventing the onset of pouchitis and improved the quality of life in patients with ileal pouch-anal anastomosis. Even though the results from the clinical trials seemed

promising in treating IBD, more large scale clinical trials are needed before VSL#3 can be prescribed to other IBD patients.

Lactose intolerance

Lactose is the main carbohydrates found in milk. Vesa and others (2000) showed that two-thirds of the world population suffers from lactose intolerance or lactose maldigestion. Lactose intolerance individuals lack or have a lower amount of β -galactosidase enzyme (De Vrese and others 2001), which functions to break down lactose into glucose and galactose. Undigested lactose draws water into the intestine and the fermentation of the lactose by gut microorganisms produces acid and gases. Consequently, bloating, cramping and diarrhea occur in lactose intolerance individuals and thus discourage them from consuming dairy products (Marteau and others 1997).

Improvement of lactose digestion in lactose intolerant individuals after the consumption of probiotics and lactose has been demonstrated by several research studies. In fact, alleviation of the discomfort in lactose intolerance individuals is probably one of the well established health claims from the consumption of *Bifidobacterium* (Scheinbach 1998). Marteau and others (1990) measured breath hydrogen content of lactose malabsorption individuals who consumed 18 g of lactose in the form of whole milk, yogurt and heated yogurt. Bacterial lactase activity was present at 1.7 IU/g in yogurt, at 0.3 IU/g in heated yogurt, and at 0 IU/g in whole milk. Results indicated that the subjects digested lactose better in the form of yogurt, followed by heated yogurt and then milk. Studies conducted by Jiang and others (1996, 1997) showed that *Bifidobacterium longum* has a positive impact on improving lactose digestion in lactose intolerant individuals. The improved lactose digestion

may be attributed to the presence of β -galactosidase in the probiotic bacteria. Bile salts in the small intestine lyse the bacteria upon ingestion and β -galactosidase contained in the probiotic bacteria are released into the small intestine to degrade lactose (De Vrese and others 2001).

Atopic diseases

Atopic diseases, such as atopic eczema, allergic rhinitis and asthma, are disorders that are commonly observed in the industrialized countries. The incidence of these allergy diseases has increased rapidly during recent decades (Kim and others 2005). In 1989, Strachan proposed the “hygiene hypothesis” that stated that with the improved hygiene in the developed countries and lack of infections in early life, children have a lower immunity and thus increased chances of atopic diseases. Several experimental studies had shown that stimulating the immune system with certain microorganisms might prevent or treat atopic diseases (Majamaa and Isolauri 1997; Matricardi and others 2003).

In a double-blind, randomized placebo controlled study conducted by Kalliomäki and others (2001), *Lactobacillus* GG was given to an expecting mother with at least one first degree relative with atopic diseases two to four weeks before delivery, and to the infants for six months after birth. The results showed that the development of atopic eczema in the probiotic group was 50% lower than the placebo group. Isolauri and others (2000) supplemented the diets of infants with atopic eczema with *Bifidobacterium lactis* or *Lactobacillus* GG and evaluated the severity of their atopic eczema with SCORing Atopic Dermatitis (SCORAD) method. After two months, significant reduction in the severity of atopic eczema was observed by comparing the SCORAD score in infants who consumed *B. lactis* (0–3.8) or *L. GG* (0.1–8.7) to the unsupplemented group (4.5–18.2).

Evidence supporting the use of probiotics in the prevention and treatment of atopic diseases are preliminary and inconclusive. Therefore, more extensive studies and clinical trials are essential.

Hypercholesterolemia

Cardiovascular diseases have been the leading causes of death and accounted for 58% and 37.3% of all deaths in the United States in 2002 and 2003 (Thom and others 2006). Hypercholesterolemia and high levels of serum cholesterol in humans, have been linked to the development of cardiovascular diseases. Diet is recognized as one way to control serum cholesterol levels. Several animal and human research studies showed that incorporating probiotic bacteria into the diet could reduce serum cholesterol (Rašić and others 1992; De Smet and others 1998; Kieβling and others 2002). Xiao and others (2003) studied serum cholesterol levels in adult male volunteers after an intake of 300mL of low fat drinkable yogurt with and without 10^8 CFU/mL *Bifidobacterium longum* supplementation for 4 weeks. A significant decrease in total cholesterol, 13.7 mg/dl, was observed in the *B. longum* group especially in subjects with moderate hypercholesterolemia compared to the placebo group and before the study ($p < 0.05$). However, some studies have showed no differences or improvement in serum cholesterol level when probiotics are given to the subjects (Rossouw and others 1981; Thompson and others 1982; De Roos and others 1999). Lin and others (1989) conducted a double-blinded, placebo-controlled study involving 354 human subjects with tablets containing 2×10^6 CFU/mL viable *L. acidophilus* and *L. bulgaricus*. Four tablets per day were consumed for six weeks, and, at the end of the study, no significant differences in serum cholesterol were observed between the treatment and placebo group.

The differences in the results among these studies may be due to the use of different strains, doses and even the subjects. The exact mechanism on how probiotics aid in reducing serum cholesterol is not fully understood. De Smet and others (1994) proposed that probiotic bacteria contain active bile acid hydrolase, which hydrolyzes conjugated bile acids, and prevents them from being reabsorbed, thus increasing the conversion of cholesterol to bile acids.

Cancer prevention

Colon cancer is the fourth most common cancer worldwide after lung, breast and prostate cancer in both men and women (Boyle and Langman 2000). Environmental and hereditary factors are the common risk factors for developing cancer. Environmental factors, such as living area, physical activity, diet and exposure to chemicals, have attracted much interest as these risk factors are controllable. Numerous results from *in vitro* studies, *in vivo* animal models studies, epidemiology and human intervention trials have presented evidence to support the prevention or treatment of colon cancer by dietary alteration and incorporation of probiotic bacteria into the diet. Researchers recommended decreased meat and fat consumption and increased dietary fiber and probiotic intake to minimize the risk of developing cancer (Brady and others 2000).

In vivo studies have supported the association of diets to the increase mutagenicity in the human body (Lidbeck and others 1992; Hayatsu and Hayatsu 1993). Ouwehand and others (2002) suggested that diets high in meat and fat or low in fiber tend to cause an increase in levels of *Bacteroides*, *Clostridium* and other putrefactive bacteria and a decrease in levels of *Bifidobacterium* in the colon. This change in microflora composition has led to an

increase in the activity of fecal enzyme, including, β -glucuronidase, azoreductase, urease, nitroreductase and glycocholic acid reductase, that could convert procarcinogens to carcinogens and thus increase the risk of colon cancer. Studies with human volunteers conducted by Lidbeck and others (1992) showed that with the consumption of *Lactobacillus acidophilus*, feces and urine mutagenicity decreased when volunteers were fed a fried meat diet, which is known to increase feces and urine mutagenicity. In another study conducted by Hayatsu and Hayatsu (1993), urine mutagenicity of the volunteers ingesting fried ground beef diet also decreased when *L. casei* was administered orally.

The incorporation of probiotics into the diets of rats has shown positive results (Reddy and Rivenson 1993; Singh and others 1997; McIntosh and others 1999; Tavan and others 2002). Tavan and others (2002) demonstrated the protective effect of milk and probiotic bacteria on the heterocyclic aromatic amine (HAA) induced colon carcinogenesis in rats. Incidence of aberrant crypts were 96% lower in rats with *B. animalis*-fermented milk supplemented diet, 93% lower with *S. thermophilus*-fermented milk supplemented diet and 66% lower in the unfermented milk supplemented diet compared with that of the control rats. Lyophilized cultures of *B. longum* were fed to rats to examine the effect of *B. longum* on inhibiting tumor induction by azoxymethane. Ingestion of *B. longum* significantly suppressed the incidence of colon tumor development and reduced tumor volume and multiplication (Singh and others 1997). Reddy and Rivenson (1993) conducted an animal model study by inducing tumors in rats with 2-amino-3-methylimidazole[4,5-f]quinoline (IQ). *B. longum* was supplemented into the male rats' diet, and the development of colon tumors was inhibited when compared to the control.

The mechanisms behind the observed effects of probiotics in preventing cancer were discussed by several researchers but it is not well defined (Commane and others 2005). More extensive and long-term studies need to be conducted to identify the precise mechanisms of action and provide the evidence for the anti-cancer effects of probiotics before their use in cancer prevention or treatment in humans can be fully justified.

Bifidobacterium

History

Bifidobacterium was originally discovered and isolated by Henry Tissier around 1899–1900 when he observed and compared feces of breast-fed infants versus bottle-fed infants. He discovered that feces of breast-fed infants have a higher number of irregular Y shaped bacterium compared to the bottle-fed infants (Kurmann and Rašić 1991). When *Bifidobacterium* was first discovered, Tissier assigned them to the *Bacillus* genera. *Bifidobacterium* was then assigned to many different genera included: *Bacteroides*, *Tissieria*, *Nocardia*, *Lactobacillus*, *Actinomyces*, *Bacterium* and *Corynebacterium* (Fooks and Gibson 2002). In 1924, Orla-Jensen proposed to name these bacteria under different genera, *Bifidobacterium*. This change was in effect in the 8th edition of Bergey's Manual, which was published in 1974 (Rogosa 1974).

Characteristics of *Bifidobacterium*

Bifidobacterium are widely found in the gastrointestinal tracts of warm-blooded animals. There are human and non-human species of *Bifidobacterium*. For humans to obtain

the health benefits of *Bifidobacterium*, it is recommended that human strains are consumed (Saarela and others 2000).

Bifidobacterium are gram positive, non-spore forming, non-gas producing and non-motile microorganisms with an irregular rod shaped appearance. *Bifidobacterium* are classified as strictly anaerobic microorganisms although their oxygen sensitivity varies among species and strains. *Bifidobacterium* that was isolated from humans have an optimum growth temperature of 36–38°C, and optimum pH between 6.5 to 7.0 (Leahy and others 2005). Acetic acid and L (+)–lactic acid, products of carbohydrate metabolism of *Bifidobacterium*, are produced in a molar ratio of 3:2.

***Bifidobacterium* species**

Bifidobacteria bifidum

B. bifidum are found in feces of infants, adults and suckling calves, and human vagina. Their shapes are highly variable rods. The bacteria are gram positive but may stain irregularly when culture ages. They have whitish, opaque, circular, convex shaped colonies with a smooth to mucoid surface. *B. bifidum* has limited fermentation ability, and they do not ferment maltose and raffinose. (Rašić and Kurmann 1983; Biavati and others 2000)

Bifidobacteria breve

B. breve are found in feces of infants and suckling calves, human vagina and sewage. They are short, slender or thick, and often club-shaped rods. The bacteria are gram positive and may autoagglutinate in saline. They have convex colonies with a diameter of 2–3 mm and smooth or wavy surface. *B. breve* do not ferment arabinose or xylose (Reuter 1971; Buchanan and Gibbon 1974; Rašić and Kurmann 1983; Biavati and others 2000).

Bifidobacteria infantis

B. infantis are found in feces of infants and suckling calves. They are usually small, thin, and often spherical or bubble-shaped rods. They often have central granules with no branching tendency. The bacteria are gram positive when stained and no clumping is observed in saline solutions. Colonies are convex, 2–3 mm in diameter, and exhibit a soft, moist consistency. *B. infantis* do not ferment arabinose, xylose, or mannitol (Reuter 1971; Buchanan and Gibbon 1974; Rašić and Kurmann 1983; Biavati and others 2000).

Bifidobacteria longum

B. longum are found in feces of infants, adults and suckling calves, human vagina and sewage. They are long, curved, club, or swollen-shaped rods. The gram stain results vary but are recognized as gram positive. No clumping is observed when treated with saline solutions. They have convex colonies with 2–5 mm in diameter and shining or slimy surface. *B. longum* ferment pentoses but not gluconate and cellobiose (Reuter 1971; Buchanan and Gibbon 1974; Rašić and Kurmann 1983; Biavati and others 2000).

Incorporation of *Bifidobacterium* into foods

Numerous clinical studies have shown that the balance of microflora population in the gastrointestinal tract is strongly associated with the overall well-being of an individual. Imbalance of the gut microflora can possibly lead to colon cancer, inflammatory bowel diseases, diarrhea and other gastrointestinal diseases (Goldin 1986; Salminen and others 1998; Saavedra 2001; Marteau and others 2002; Guarner and Malagelada 2003; Servin 2004). However, maintaining the balance of the microflora population in the gastrointestinal tract has become challenging as our lifestyles have changed with the development of technology.

Increases in stress, which consequently increase the demand on the immune system, can disrupt the equilibrium of the gut microflora. In addition, changes in the diet and living conditions can alter the gut microflora and affect their overall functionality. Antibiotic treatment, which destroys the balance of the gut microflora, can increase the chances of infection and colonization by pathogenic microorganisms in the gastrointestinal tract (Rambaud and others 1993). The combination of all these factors can shift the balance of the gut microflora and thus lead to gastrointestinal-tract related disorders. Therefore, maintaining the balance and health of the gut microflora by increasing the number of beneficial or health-promoting bacteria, such as *Bifidobacterium*, in the gastrointestinal tract is essential. Incorporation of *Bifidobacterium* into the diet has been shown to effectively increase the number of health-promoting microorganisms in the gut and prevent the overgrowth of pathogenic microorganisms (Tannock 2002).

Nowadays, products with *Bifidobacterium* are widely available in the market. Freeze-dried *Bifidobacterium* is available in most health food stores or pharmacies as supplements in tablets, capsules or powders. A variety of food products supplemented with *Bifidobacterium* are obtainable from the grocery stores, including yogurt, milk, fermented milk, fruit juices and different types of cheeses (Ishibashi and Shimamura 1993; Scheinbach 1998; Chandan 1999). However, due to the growth requirements of *Bifidobacterium*, not all food systems are suitable to be used as carriers for the delivery of *Bifidobacterium* to humans. There are several challenges that need to be overcome in order to deliver an effective therapeutic dose of *Bifidobacterium* to the consumer via food.

Challenges

The major challenges in incorporating *Bifidobacterium* into food products and deliver their beneficial effects to the consumers are the viability of the bacteria over the storage period or the shelf life of the food, their survivability during gastrointestinal tract transit and their ability to colonize the gastrointestinal tract to provide the health benefits to the host (Saarela and others 2000; Ross and others 2005). The recommended beneficial level or the therapeutic dose of *Bifidobacterium* has not yet been precisely determined but is estimated to be between 10^7 CFU/g foods to provide the health benefits to the consumer (Ross and others 2005). Thus, the concentration of the bacteria has to be maintained at this level or above over the storage period and the shelf life of the food to be effective (Rambaud and others 1993). In addition, some studies had demonstrated that some strains of *Bifidobacterium* do not survive well during the transit in the gastrointestinal tract. The acidity of the gastric juice and the antimicrobial action of the bile salts can cause damage to the *Bifidobacterium* (Ibrahim and Bezkorovainy 1993; Marteau and others 1993; Lankaputhra and Shah 1995; Charteris and others 1998).

Product and Storage Stability

Bifidobacterium are incapable of oxygen respiration or growth under aerobic condition and thus, they are classified as strict anaerobes (Scardovi 1984). This characteristic has become a challenge to the industry when trying to incorporate *Bifidobacterium* into foods. Prevention of oxygen toxicity to the microorganisms during the manufacturing and the storage of the food is crucial to deliver foods with viable cells to the consumer. However, the sensitivity to oxygen is different between species and between different strains within a species (Biavati and others 2000; Talwalkar and Kailasapathy 2003). Some species or strains

of *Bifidobacterium*, for instance, *B. infantis*, *B. longum*, *B. breve*, *B. lactis* and *B. pseudolongum* are able to tolerate oxygen by limiting their metabolic activity as shown by the reduced production of lactic acid under aerobic conditions (Shimamura and others 1992; Meile and others 1997; Talwalkar and Kailasapathy 2003). Therefore, selection of the strains or species that can tolerate higher oxygen stresses is favorable in order to improve the viability of *Bifidobacterium* after incorporation into food and during the storage and shelf life of the food.

Most species of the *Bifidobacterium* isolated from human origin have an optimum growth temperature of 36–38°C, whereas animal strains have a slightly higher optimum growth temperature of 41–43°C. Even though the optimal growth temperature is between 36–43°C, a slower growth rate can be observed at a wide temperature range from 25–28°C and 43–45°C (Scardovi 1984). Some strains can grow at higher or lower temperature for instance, *B. thermacidophilum* and *B. psychraerophilum* which grow at 49.5°C and 4°C, respectively (Dong and others 2000; Simpson and others 2004).

With the wide range of growth temperatures observed for *Bifidobacterium*, the storage temperature of the food is crucial. If the food is stored at room temperature or higher, growth of *Bifidobacterium* in the food might occur and the production of metabolites, such as lactic acid and acetic acid may affect the sensory quality of the food (Modler and others 1990). In addition, foods that require thermal processes will require an additional step to incorporate *Bifidobacterium* into the food because *Bifidobacterium* is not a thermoresistant microorganism and heating at high temperature will destroy the microorganism (Rašić and Kurmann 1983). Without viable cells, the therapeutic effects of consuming the functional food cannot be exercised.

The optimal pH for the growth and survivability of *Bifidobacterium* is approximately 6.5 and 7.0. There is little or no growth observed below the pH range of 4.5 to 5.0 or above 8.0 to 8.5 (Scardovi 1984), and *Bifidobacterium* do not survive at pHs above 8.5 (Biavati and others 2000). However, the acid tolerance of *Bifidobacterium* is strain and species specific as well. In milk acidified with lactic acid to pH 4.0, no significant losses in viability of *Bifidobacterium bifidum* and *B. longum* BL cells were observed after 4 weeks of storage at 5°C (Vinderola and others 2002). Some strains of *Bifidobacterium* including *B. animalis* and *B. lactis* survived exposure to pH 3.5 (Matsumoto and others 2004). Nevertheless, the inability to grow at lower pHs has limited the use of *Bifidobacterium* as a starter culture to ferment dairy products that have a low final pH, for instance, yogurt, which has a pH between 3.7 and 4.3. During the fermentation process, *Bifidobacterium* utilizes the carbohydrates in the food and produces acetic acid and lactic acid. The production of the acids decreases the pH in the food system and thus inhibits the growth of *Bifidobacterium* to halt the fermentation process. However, studies had shown that the survivability and growth of *Bifidobacterium* could be improved by acid-adaptation. Acid-adapted *B. breve* was shown to have a greater survivability compared to non-adapted cells (Park and others 1995).

Gastrointestinal Tract Transit

In order to obtain the beneficial therapeutic effects from *Bifidobacterium*, the microorganisms have to be able to survive the transit through the gastrointestinal tract and reach the colon in high enough quantities to facilitate colonization in the gut (Saarela and others 2000). The acidity of the gastric juice and the anti-bacterial action of bile salts are critical to the viability of the *Bifidobacterium* during the gastrointestinal tract transit (Dunne and others 1999). The pH of the gastric juice normally ranges from 1.0 to 3.0, which is

unfavorable for the survivability of most microorganisms. The concentration of the bile acids in bile usually ranges from 0.06% to 2.0%, and bile is harsh on the viability of most microorganisms as well (Ibrahim and Bezkorovainy 1993). Results varied when *in vitro* and *in vivo* tests were performed on the viability of *Bifidobacterium* after exposure to stimulated gastric juice and bile solutions. *Bifidobacterium* strains, such as *B. longum* 1941 and *B. pseudolongum* 20099, used in a study conducted by Lankaputhra and Shah (1995) showed great survivability after treatment with simulated gastric juice and bile salts but *B. thermophilum* 20210 used in their study did not survive as well when the similar treatment was applied. Survival studies conducted by other researchers also showed that some *Bifidobacterium* strains survived better than other strains when treated with gastric juice and bile solutions (Berrada and others 1991; Clark and others 1993; Clark and Martin 1994; Chung and others 1999; Matsumoto and others 2004). Therefore, *Bifidobacterium* strains that are able to tolerate the low pH of the stomach and the antimicrobial effects of bile salts produced by the small intestines are preferred for use as dietary adjuncts in food.

To permanently establish *Bifidobacterium* in our gut, the ability of the bacteria to adhere to the intestinal surface mucosal cells is essential (Guarner and Schaafsma 1998). Adhesion to the intestinal tract allows *Bifidobacterium* to colonize and grow, and thus shift the balance of the microflora in the gut to provide the beneficial therapeutic effects to the host. In addition, only adhered bacteria have been thought to effectively induce immune effects and stabilize the intestinal mucosal barrier through contact with the gut associated lymphoid tissue (Bezkorovainy 2001; Dunne and others 2001). Therefore, *Bifidobacterium* strains with high adhesion properties are preferable because the average survival rate of the bacteria after the transit in our gastrointestinal tract has been estimated to be around 30%

(Bouhnik and others 1992). Several *in vitro* studies showed that *Bifidobacterium* are able to adhere to the intestinal mucosal cells however, the adhesion potentials are different among different strains (Kirjavainen and others 1998; Pérez and others 1998; Tuomola and Salminen 1998). For example, *B. longum* 16 and *B. breve* 4 used in the study by Bernet and others (1993) showed a better adhesion property to human intestinal epithelial Caco-2 cells compared to *B. bifidum* 8 used in the study.

***Bifidobacterium* growth factors**

In order to obtain the therapeutic beneficial effects of *Bifidobacterium*, it is necessary that this microorganism survives and grows in the lower gastrointestinal tract. Therefore, *Bifidobacterium* growth factors are commonly added to the food to improve the survivability and growth of the bacteria. Whey protein concentrate and fructooligosaccharide are common growth factors that are incorporated into food with *Bifidobacterium* to enhance the growth and viability of the bacteria in the food.

Whey Protein Concentrate

Whey protein concentrate (WPC) is an inexpensive and readily available source of nutrients generated from the cheese production industry. WPC contains caseinomacropeptide, a hydrophilic glycopeptide that contains available nitrogen and amino-sugars that could be fermented by *Bifidobacterium*. In a study conducted by Petschow and Talbott (1990), whey fraction of cow's milk and human milk were compared and results indicated that the whey fraction of cow's milk promotes the growth of *Bifidobacterium* as effectively as the whey fraction from human milk. Bury and others (1998) showed that with the addition of WPC as a nutrient supplement in the whey broth fermentation process, the growth of the probiotic

bacteria was higher compared to the control with no WPC supplemented. Numerous research studies have shown that with the incorporation of WPC into the fermented milk and yogurt, the viability and growth of *Bifidobacterium* is improved (Janer and others 2004; Martín–Diana and others 2003). Sodium dodecyl sulfate–PAGE and amino acid analyses performed by Dave and Shah (1998) suggested that the nitrogen source in the form of peptides or amino acids from the WPC improved the viability of *Bifidobacterium* when WPC is added into yogurt fermentation.

Fructooligosaccharides

Fructooligosaccharides (FOS) are natural carbohydrates that are found in a wide variety of fruits, vegetables and cereals (Losada and Olleros 2002). The human body does not produce enzymes that can digest FOS. Thus, FOS is classified as a non–digestible oligosaccharide as well as dietary fiber (Molis and others 1996; Roberfroid 2000a). Undigested FOS transit from the upper gastrointestinal tract into the intestine and colon, and *Bifidobacterium* that are present in the intestine and colon utilize FOS through fermentation (Collins and Gibson 1999). FOS has been demonstrated as an effective *Bifidobacterium* growth promoting factors through both *in vivo* and *in vitro* assessments. Several *in vivo* studies showed that the incorporation of 8–15g of FOS in the diet increases *Bifidobacterium* counts in the feces (Gibson and others 1995; Guigoz and others 2002; Boehm and others 2005). *In vitro* studies by Alkalin and others (2004) also showed that with the addition of 2% FOS, the viability and growth of *Bifidobacterium animalis* and *B. longum* were enhanced in the fermentation process for the production of yogurt. Similar trends were observed in the production of fermented milk and batch culture fermentation when FOS is added (Gibson and Wang 1993; Bruno and others 2002).

Incorporation of *Bifidobacterium* into yogurt and fermented milk

Dairy products are one of the most commonly available foods to function as the carrier for the delivery of *Bifidobacterium* into the human gut. Milk, fermented milk, yogurt and cheeses with *Bifidobacterium* are currently available in the market. The buffering capacity of milk protein has helped to ensure the survivability of *Bifidobacterium* during the transit in the upper gastrointestinal tract and the low levels of oxygen in milk can promote a favorable environment for the survival of the strict anaerobic *Bifidobacterium* (Klaver and others 1993; Charteris and others 1998; Chandan 1999). Thus, the interest of utilizing dairy products as vehicles for transferring the probiotics into the human gut has increased and extensive research has been performed.

There is a wealth of research focused on the incorporation of *Bifidobacterium* into yogurt and fermented milk. *Bifidobacterium* are added into fermented milk and yogurt either with the starter culture before the fermentation process or after the fermentation process. The addition of *Bifidobacterium* into the milk before the fermentation process is critical because the bacteria may alter the fermentation process and the quality of the final product. In addition, the decrease in pH of the yogurt and fermented milk during the fermentation process may decrease the viability of the *Bifidobacterium* in the end product. Besides these factors, during low temperature storage, the viability of the *Bifidobacterium* is affected by the low pH of the fermented milk and yogurt (Klaver and others 1993). Therefore, selections of *Bifidobacterium* strains that can tolerate low pH environment are favorable.

Bifidobacterium is a strict anaerobe and the oxygen levels in yogurt and fermented milk may decrease the viability of the bacteria. Ishibashi and Shimamura (1993) have developed a solution to ensure the viability of *Bifidobacterium* during the storage and shelf

life of the yogurt. They reported that *Streptococcus thermophilus* utilizes a large amount of oxygen and thus *Bifidobacterium* can be inoculated with *S. thermophilus*. The oxygen present in the yogurt is then utilized by *S. thermophilus* and hence the viability of *Bifidobacterium* is enhanced.

In addition to selecting the *Bifidobacterium* strains that survive best in the fermented milk and yogurt, other techniques have been utilized to enhance the viability and growth of *Bifidobacterium* in the cultured dairy products. The addition of supplements, for instance, amino acids, peptides, prebiotics and other micronutrients to increase the survivability and growth of the bacteria have been extensively studied. Ascorbic acid and cysteine were added into yogurt and fermented milk to decrease the redox potential in the food and provide a more favorable environment for the growth of *Bifidobacterium* (Klaver and others 1993; Dave and Shah 1997a, b). However, in the presence of the yogurt cultures, *L. delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*, and the fermented milk cultures, *L. acidophilus* Ki and *S. thermophilus* S3, the viability of the *Bifidobacterium* was not improved by the addition of ascorbic acid and cysteine in these studies. The yogurt and the fermented milk cultures decreased the pH in the milk before the favorable redox potential for the *Bifidobacterium* was achieved (Klaver and others 1993).

Production of yogurt supplemented with whey protein concentrate, acid casein hydrolysate or tryptone enhanced the viability of *Bifidobacterium* through the addition of nitrogen in the form of peptides and amino acids (Dave and Shah 1998). Supplementation of fermented goat's milk with whey protein concentrate decreased the fermentation time by 2 hours and increased the viable counts of *Bifidobacterium* by 0.7 log units (Martín-Diana and others 2003). Addition of prebiotics, for instance 2% FOS, into yogurt maintained the

viability of *B. longum* above 10^6 CFU/mL for 21 days storage time compared to 7 days in the control with no FOS supplemented (Alkalin and others 2004).

The incorporation of *Bifidobacterium* into other food products has been studied and several different approaches have been examined to increase their viability in food and during gastrointestinal tract transit. More clinical studies are needed to compare the survivability of *Bifidobacterium* in different food products after the gastrointestinal tract transit.

MATERIALS AND METHODS

Bifidobacterium Culture Preparation

Freeze-dried *Bifidobacteria bifidum* R0071, *B. breve* R0070, *B. infantis* R0033 and *B. longum* R0175 cultures obtained from Institut Rosell/Lallemand (Montreal, Canada) were used throughout the experiments. Freeze-dried powdered cultures were added to 30 mL MRSC broth, which is prepared by supplementing MRS broth (Difco Laboratories, Detroit, MI, USA) with filter-sterilized 0.05% L-cysteine hydrochloride (Sigma Chemical Co., St. Louis, MO, USA) and 0.02% resazurin (Sigma Chemical Co., St. Louis, MO, USA). L-cysteine was added to MRS broth 24 hours prior to inoculation of the culture to achieve reduced conditions in the broth medium for the optimal growth of *Bifidobacterium*. Resazurin was added as an oxygen indicator. In order to achieve a strict anaerobic environment, the headspace in each tube of MRSC broth with inoculated *Bifidobacterium* was purged for 30 seconds with nitrogen filtered through glass wool. The purged tubes were sealed with butyl rubber stoppers and wrapped with parafilm prior to incubation at 37°C for 24 hours.

After 24 hours, 1 mL of each culture, containing approximately 10^9 CFU/mL, was transferred aseptically into fresh 30 mL MRSC broth, purged with nitrogen and sealed with butyl rubber stopper and parafilm. The cultures were incubated at 37°C for 20 hours. Gram stain was performed and the characteristics of the bacteria were identified microscopically. After 20 hours, 1 mL of each culture was transferred into 200 mL of fresh MRSC broth, nitrogen purged, sealed with parafilm and incubated at 37°C for 20 hours.

Preparation of Samples

Cells were harvested by centrifugation (Sorvall, Newton, CT, USA) at 10,000 X g for 10 minutes at 4°C. Supernatant was discarded and pellets of harvested cells were washed by suspending the pellet into 200 mL of sterile 0.85% saline solution. Saline solution was removed after centrifugation (10,000 X g for 10 minutes at 4°C) of the cell suspension. Pellets of *Bifidobacterium* cells were then suspended into 200 mL of commercially available 2% milk (HyVee, Des Moines, IA, USA). The final concentration of *Bifidobacterium* obtained was approximately $9 \log_{10}$ cfu/mL.

In order to obtain 200 mL of $8 \log_{10}$ CFU/mL *Bifidobacterium* milk sample, 20 mL of $9 \log_{10}$ CFU/mL *Bifidobacterium* milk samples were aseptically transferred into 180 mL of fresh milk. Different inoculation levels were prepared by diluting $9 \log_{10}$ CFU/mL *Bifidobacterium* milk samples with the appropriate amount of milk aseptically. Separate samples were prepared for each sampling day. Samples were stored in 4°C walk-in cooler.

Preparation of Supplements

Whey protein concentrate (WPC; Instantized WPC 8010, Hilmar Ingredients, Oakland, CA, USA) and fructooligosaccharides (FOS; Beneo™ P95, ORAFTI, Malver, PA, USA) were vacuum packaged and sterilized by irradiation at 40 kiloGray. Stock solutions of WPC and FOS were prepared by dissolving 70 g of each supplement in 280 mL of milk (w/v) aseptically. In order to prepare a 2% supplemented samples, 16 mL of the supplement were added into 184 mL *Bifidobacterium* milk samples; 24 mL of the supplement were added into 176 mL *Bifidobacterium* milk samples to prepare a 3% supplemented samples. Samples were

inverted 3 times to obtain homogeneous samples. Separate samples were prepared for each sampling day. Samples were stored in 4°C walk-in cooler.

Simulated Gastric Juice and Bile Solutions Preparation

Simulated gastric juice and bile solutions were prepared according to Lian and others (2003). In this study, pH 3.0 was selected for gastric juice and 1% concentration was selected for bile solutions.

Simulated gastric juice was prepared by suspending 1.5 g of pepsin (Fisher Scientific, Fairlawn, NJ, USA) in 500 mL of 0.5% saline solution. The simulated gastric juice was adjusted to pH 3.0 with 12 N hydrochloric acid (HCl) (Fisher Scientific, Fairlawn, NJ, USA) and sterilized by membrane filtration (0.45 µm) (Fisher Scientific, Fairlawn, NJ, USA). Sterilized simulated gastric juice (10 mL) was pipetted aseptically into sterilized cap tubes.

Bile solutions (1%) were prepared by suspending 1 g of oxgall (Difco Laboratories, Detroit, MI, USA) into 99 ml of distilled water. Bile solution (10 mL) was pipetted into cap tubes and sterilized by autoclaving at 121°C for 15 minutes. Both simulated gastric juice and bile solutions tubes were stored at 37°C prior to the experiments.

Microbial Analysis

Survivability of *Bifidobacterium* in milk

Survivability of *Bifidobacterium* in milk samples were determined at 3-days intervals throughout the 15-day storage period. At each sampling day, 1 bottle of the milk samples was removed from the walk-in cooler and inverted 20 times to obtain a homogeneous sample. One mL of the milk samples was serially diluted with 9 mL of sterilized 0.1% peptone water

to obtain dilutions ranging from 10^{-2} to 10^{-7} . Samples of appropriate dilutions were spread plated on MRSC agar (MRS agar with 0.05% L-cysteine hydrochloride). Control milk samples with no *Bifidobacterium* inoculated were plated on MRSC to determine the counts of background microflora on each sampling day.

Each sample with appropriate dilutions was spread plated in duplicate. Plates were incubated in anaerobic jars with AnaeroGenTM 3.5 L gas packs (Oxoid, Inc., Basingstroke, Hampshire England) at 37°C for 72 hours. Colonies on each plate were counted, averaged, and expressed as log colony-forming units per mL sample (log CFU/mL). Colonies were gram stained and analyzed microscopically to confirm the identity of the bacteria.

Survivability of *Bifidobacterium* in simulated gastrointestinal tract transit

Simulated gastrointestinal tract transit survivability assays were performed on *Bifidobacterium* milk samples on days 0, 6, 12 and 15 by using simulated gastric juice and bile solutions. Ten mL of *Bifidobacterium* milk sample was added into cap tubes containing 10 mL of sterilized simulated gastric juice. Samples were vortexed and incubated in a 37°C water bath for 4 hours. After 4 hours, samples were vortexed and serial dilutions were performed with 9mL of sterilized 0.1% peptone water. Cell enumerations were determined by using the spread plate method on MRSC agar. Appropriate dilutions were used and plates were incubated at 37°C for 72 hours anaerobically. The survivability of *Bifidobacterium* in the bile solutions was tested by using the same procedure conducted for the simulated gastric juice. The survivability of *Bifidobacterium* during simulated gastrointestinal tract transit was expressed as % survivability by dividing the count after 4 hours with the count at the day of analysis and multiplied by 100%.

Lactose Content

Lactose content was measured at 3-day intervals throughout the 15-day storage period. Megazyme lactose and D-galactose (rapid) assay kits (Megazyme International Ireland Ltd., Bray, Ireland) were used, and milk without *Bifidobacterium* was used as a control. Samples were extracted by adding 1 mL of milk samples into 100 mL volumetric flask containing 60 mL distilled water. Samples were held at 50°C with occasional swirling for 15 minutes. Carrez I solution (2 mL) and Carrez II solution (2 mL) were added with mixing after each addition. Sodium hydroxide solution (4 mL, 100 mM) was then added and mixed vigorously. Samples were diluted to the mark with distilled water and mixed. Whatman No. 1 filter papers were used to filter the samples. The first few mL of the samples were discarded, and the clear filtrate was used in the assay.

Samples were treated with β -galactosidase to hydrolyze the lactose into D-glucose and D-galactose. Galactose mutarotase were added to interconvert α -D-galactose to β -D-galactose. β -galactose dehydrogenase and NAD^+ were added to oxidize β -D-galactose to D-galactonic acid and form NADH. The amount of NADH formed (mol) is equivalent to the amount of lactose present in the samples. The concentration of NADH was measured by using a spectrophotometer (Spectronic Genesys 10, Thermo Electron Co., Waltham, MA, USA) at 340 nm wavelength. Duplicate measurements were conducted for each sample. Lactose content of the samples was calculated and results were expressed as gram lactose/mL milk.

Statistical Analysis

The experiments were designed as 2–way factorials with treatment and storage time as the main factors. The experiments were conducted in duplicate. Data were analyzed by analysis of variance (ANOVA) using Statistical Analysis System software program's (SAS Institute Inc., Cary, NC) General Linear Model (GLM) to determine the significance of main factors and interactions between main factors. Differences between factors were determined using Tukey's honestly significant differences test. The level of significance was set at $p < 0.05$.

Study 1: Survivability of *Bifidobacterium* strains in milk

The experiment was designed as a 2–way factorial with the *Bifidobacterium* strains, *B. bifidum* R0071, *B. breve* R0070, *B. infantis* R0033 and *B. longum* R0175 and storage time as the main factors. Each *Bifidobacterium* strain was inoculated at 10^8 CFU/mL and stored for 15 days in 4°C walk–in–cooler. Viability of the *Bifidobacterium* strains in milk and after 4 hour–simulated gastrointestinal tract transits were studied. Lactose content was measured.

Study 2: Effect of inoculation levels on the survivability of *B. bifidum* R0071 in milk

B. bifidum R0071 was selected for this experiment and inoculated into milk at 3 different inoculation levels, 10^4 , 10^5 and 10^6 CFU/mL. Viability over the storage period and after the 4 hour–simulated gastrointestinal tract transit was studied. Effect of inoculation level and the storage time on viability of the *B. bifidum* R0071 was analyzed in this experiment.

Study 3: Effect of supplements on the survivability of *B. bifidum* R0071 in milk

B. bifidum R0071 was inoculated into the milk at 10^6 CFU/mL. Samples were supplemented with 2 or 3% WPC or FOS. Supplements and storage time were the main factors in this experiment. *B. bifidum* R0071 viability over the storage time and after the 4 hour-treatment with simulated gastrointestinal tract transit was studied. The effects of the treatment and the storage time on the viability of the *B. bifidum* R0071 were analyzed.

RESULTS

Milk samples without *Bifidobacterium* were plated on MRSC agar and incubated anaerobically at 37°C for 72 hours on each sampling day to determine the counts of background microflora. The background microflora in these three studies throughout the 15-day sampling time was less than 10^2 CFU/mL (results not shown). The amount of *Bifidobacterium* present in the samples in each study was greater than 10^4 CFU/mL. The dilutions performed in these studies diluted out the background microflora that might be present in the milk samples. Therefore, the counts obtained from the *Bifidobacterium* milk samples throughout the studies were not affected by the background microflora.

Study 1: Effect of *Bifidobacterium* Strains on Viability in Milk and during Gastrointestinal Tract Transit

Survivability of the four *Bifidobacterium* strains, *B. infantis* R0033, *B. longum* R0175, *B. breve* R0070 and *B. bifidum* R0071 during the 15-day storage period at refrigeration temperature, 4°C, is illustrated in Figure 1. The bacteria were inoculated at approximately $8 \log_{10}$ CFU/mL into 2% fat milk. Satisfactory cell survivability with no log reduction greater than 1 \log_{10} CFU/mL was observed for all the *Bifidobacterium* strains used in this study over the 15-day storage period. The interaction between the strains and the storage time was not significant. The log reduction at day 15 compared to day 0 for the four *Bifidobacterium* strains, *B. infantis* R0033 (0.57 \log_{10} CFU/mL), *B. longum* R0175 (0.20 \log_{10} CFU/mL), *B. breve* R0070 (0.32 \log_{10} CFU/mL), and *B. bifidum* R0071 (0.11 \log_{10} CFU/mL) was not significant ($p < 0.05$).

When comparing the effect of the strains at each sampling day, at days 6 and 12, *B. breve* R0070 has a significantly higher count compared to *B. infantis* R0033, and, at day 15, both *B. breve* R0070 and *B. longum* R0175 had significantly higher counts than *B. infantis* R0033 ($p < 0.05$). The higher count observed in *B. breve* R0070 and *B. longum* R0175 can be explained by the slightly higher inoculation level at day 0 for *B. breve* R0070 ($8.69 \log_{10}$ CFU/mL) and *B. longum* R0175 ($8.52 \log_{10}$ CFU/mL) when compared to *B. infantis* R0033 ($7.93 \log_{10}$ CFU/mL) although these inoculation levels were not significantly different than one another. The more pronounced reduction in the log count observed in *B. infantis* R0033 over the storage time might contribute to the differences observed at day 12 and 15 as well.

Figure 2 shows that *Bifidobacterium* strains used in this study survived after 4 hours treatment with simulated pH 3.0 gastric juice at 37°C. Survivability following the simulated gastric juice treatment is compared to the counts in the milk for that sampling day. The interaction between the storage time and the strains was significant ($p < 0.05$). When comparing the effect of the storage time for each strain, *B. breve* R0070 showed significantly better survival in simulated gastric juice at day 12 and 15, with 110.39% survivability and 110.11% survivability respectively, compared to day 0 (102.21%) and 6 (101.61%). No significant differences in the survivability were observed in *B. infantis* R0033, *B. longum* R0175 and *B. bifidum* R0071 over the storage period. When the effect of the strains was compared on each sampling day, similar survivability rate were observed at each sampling day among each strains. For example, at day 0, no significant differences ($p < 0.05$) were observed among each strain and the same trend was observed at day 6, 12 and 15.

Survivability of *Bifidobacterium* strains after 4 hours of treatment with 1% (w/v) simulated bile solutions in 37°C water bath is illustrated in Figure 3. The interaction between

the storage time and the strains was significant ($p < 0.05$). When comparing the effect of storage time on the percent survivability of each *Bifidobacterium* strains, at day 15, survivability for *B. bifidum* R0071 (91.42%) was reduced significantly compared to day 0 (99.45%), 6 (101.03%) and 12 (100.89%) after the treatment with simulated bile solutions. Survivability for *B. infantis* R0033, *B. longum* R0175 and *B. breve* R0070 were not significantly different over the 15 days storage period ($p < 0.05$). Differences were observed at day 15 when comparing the strains effect on each sampling day. At day 15, *B. bifidum* R0071 (91.42%) showed a significantly lower percent survivability compared to *B. infantis* R0033 (101.08%), *B. longum* R0175 (101.31%) and *B. breve* R0070 (104.56%), which was not observed at day 0, 6 and 12.

Lactose content in the *Bifidobacterium* milk samples were measured over the storage period. Fresh milk samples were used as control and the results are shown in Figure 4. The interaction between the strains and the storage time was significant ($p < 0.05$). The relatively straight line observed from Figure 4 for lactose content in control, *B. infantis* R0033, *B. longum* R0175 and *B. breve* R0070 samples showed that lactose content was maintained over the storage period in these samples. This result indicates that *B. infantis* R0033, *B. longum* R0175 and *B. breve* R0070 did not hydrolyze lactose when they were inoculated into milk and stored at 4°C for 15 days. A different trend was observed in milk samples inoculated with *B. bifidum* R0071. At day 3, the lactose content in *B. bifidum* R0071 milk was slightly lower than other samples and from day 6 to day 15, the lactose content in *B. bifidum* R0071 was significantly lower than the other treatments ($p < 0.05$).

Bifidobacterium bifidum R0071 is the only *Bifidobacterium* strains used in this study that expressed lactase activity. This indicated that, with the incorporation of *B. bifidum*

R0071 into milk, the discomfort in lactose maldigestion individuals might be alleviated. Therefore, to increase the therapeutic effects obtained from consumption of *Bifidobacterium*, incorporation of *B. bifidum* R0071 into milk compared to other *Bifidobacterium* strains used in this study is preferable.

Inoculation of *Bifidobacterium* at $8 \log_{10}$ CFU/mL showed a relatively stable survivability over the storage period. During the simulated gastric juice treatment, growth was observed in the *Bifidobacterium* milk samples. The growth observed was not greater than $1 \log_{10}$ CFU/mL. This might be attributed to the relatively high inoculation level used in this study that hinders the growth of *Bifidobacterium*. Therefore, in the next study, inoculation at a lower level was examined to observe the effect of inoculation level on the survivability of *Bifidobacterium bifidum* R0071 in the milk over the 15-day storage period and their survivability and growth during the simulated gastrointestinal tract transit treatment.

Study 2: Effect of Inoculation Levels on Viability of *B. bifidum* R0071 in Milk and during Gastrointestinal Tract Transit

The interaction between the inoculation levels and the viability over the storage period was not significant over the 15-day storage time and after the treatment with simulated gastrointestinal tract transit ($p < 0.05$).

Figure 5 shows the survivability of *B. bifidum* R0071, inoculated into milk at 4, 5, and $6 \log_{10}$ CFU/mL, over the 15-day storage period. The relative log counts for each sampling days compared to day 0 for each sample was determined. No significant differences were observed among each sample at each sampling day ($p < 0.05$). The increase or decrease in the survivability of *B. bifidum* R0071 inoculated at 3 different levels over the 15-day

storage period was not greater than 10%, which is approximately $0.5 \log_{10}$ CFU/mL, and was not significant. The inoculation levels for *B. bifidum* R0071 used in this study did not affect their survivability in milk significantly over the storage period ($p < 0.05$).

Survivability after simulated gastrointestinal tract transit is presented in Figure 6 and 7 for *B. bifidum* R0071 inoculated at 3 different concentrations. As shown in Figure 6, after 4 hours of treatment with pH 3.0 gastric juice, the survivability of *B. bifidum* R0071 was not affected and slight growth was observed for all treatments at each sampling day. No significant differences were observed among each treatment at each sampling day. Storage time did not affect the simulated gastric juice survivability of each treatment significantly as well ($p < 0.05$).

Survivability of *B. bifidum* R0071 (Figure 7) inoculated at 3 different concentrations after simulated bile solutions treatments were similar to the results observed in the simulated gastric juice treatment. No significant differences were observed among treatments over the storage period and at each sampling day ($p < 0.05$).

Even though the samples were inoculated at lower concentrations compared to the first study, no significant growth was observed after treatment with simulated gastric juice and bile solutions at 37°C for 4 hours ($p < 0.05$). *B. bifidum* R0071 used in this study survived the simulated gastrointestinal tract treatment but was not capable to grow in the environment even though their optimal growth temperature was presented.

The changes in the log count observed over the storage period and after treatment with simulated gastric juice and bile solutions were less than 10%, which is, $1 \log_{10}$ CFU/mL. Thus, inoculation at the minimal therapeutic dose, $6 \log_{10}$ CFU/mL, into the milk may be sufficient to provide the consumer with the acclaimed health benefits since they survived the

storage and gastrointestinal tract transit in a biologically effective dosage. Therefore, in the next study, *B. bifidum* R0071 is inoculated at $6 \log_{10}$ CFU/mL and supplements, whey protein concentrate (WPC) and fructooligosaccharides (FOS), were used to examine their effects on the survivability of *B. bifidum* R0071 during storage and simulated gastrointestinal tract transit.

Study 3: Effect of Supplements on the Survivability of *B. bifidum* R0071 in Milk and during Gastrointestinal Tract Transit

The effects of supplements, WPC and FOS at 2% and 3%, compared to the control on the survivability of *B. bifidum* R0071 over the 15-day storage period are presented in Figure 8. The interactions between the samples and the storage time were significant ($p < 0.05$). The control, WPC 2% (WPC2) and WPC 3% (WPC3) samples exhibited no significant changes in the log count during the 15-day storage period. However, the survivability of samples supplemented with 2% FOS (FOS2) or 3% FOS (FOS3) decreased during the storage time and at day 12 ($5.44 \log_{10}$ CFU/mL, $5.25 \log_{10}$ CFU/mL) and 15 ($5.11 \log_{10}$ CFU/mL, $5.22 \log_{10}$ CFU/mL), the viable count of *B. bifidum* R0071 in FOS2 and FOS3 were significantly lower than their inoculated level ($6.13 \log_{10}$ CFU/mL, $6.14 \log_{10}$ CFU/mL).

At each sampling day, no significant differences were observed among control, WPC2 and WPC3. At day 12 and 15, FOS2 and FOS3 showed a significantly lower survivability compare to the control, WPC2 and WPC3 samples ($p < 0.05$). Reduction of approximately $1 \log_{10}$ CFU/mL were observed in both FOS2 and FOS3 samples over the 15-day storage period.

Interactions between the supplements and the *B. bifidum* R0071 viability over the storage period after the simulated gastrointestinal tract transit treatment were not significant ($p < 0.05$). Supplementation with WPC and FOS did not affect the survivability of *B. bifidum* R0071 significantly in the simulated gastric juice, as shown in Figure 9 ($p < 0.05$). All samples survived the simulated gastric juice treatment and no significant differences were observed among each sample over the storage period. WPC and FOS used in this study did not promote growth of *B. bifidum* R0071 when treated with simulated gastric juice at their optimal growth temperature, 37°C.

Effects of supplements on the survivability of *B. bifidum* R0071 in the simulated bile solutions are shown in Figure 10. Over the storage period, the effect of storage time did not affect the survivability of *B. bifidum* R0071 during simulated bile solutions treatment and no significant differences were observed ($p < 0.05$). When comparing the effect of treatments at each sampling day, at day 0, 6 and 12, the survivability toward simulated bile solutions treatment were not different significantly. At day 15, FOS2 samples showed a significantly better survival compare to the control samples with no supplements added. Even though differences were observed at day 15 among the samples, the differences were not greater than 11%, which is less than 1 log₁₀ CFU/mL.

DISCUSSION

Effect of *Bifidobacterium* Strains and Inoculation Levels in Milk

Storage stability

As shown in Figure 1, no significant growth or reduction was observed for all the *Bifidobacterium* strains used in this study when inoculated into milk at approximately $8 \log_{10}$ CFU/mL and stored at 4°C for 15 days ($p < 0.05$). Similar results were observed for *B. bifidum* R0071 inoculated at 4, 5 and $6 \log_{10}$ CFU/mL (Figure 5). The storage temperature, 4°C, used in this study is not the optimum growth temperature for *Bifidobacterium*, therefore, no growth would be expected during the storage period (Leahy and others 2005). Survival rather than growth is desirable because when *Bifidobacterium* grows, metabolites, such as lactic acid and acetic acid, will be produced (Rašić and Kurmann 1983; Modler and others 1990). The production of acids might alter the pH of the milk and consequently affect the sensory properties of the milk. Reduction in counts of *Bifidobacterium* over the storage period is not desirable as well. If the *Bifidobacterium* lose their viability over the storage period, the milk might not be able to deliver a sufficient amount of *Bifidobacterium* to the consumer to achieve the required therapeutic dose, which is greater than $6 \log_{10}$ CFU/mL (Kurmann and Rašić 1991).

The results obtained from these studies (Figure 1 and Figure 5) agreed with those of Hughes and Hoover (1995), who found that cultures of *B. bifidum*, *B. longum*, *B. breve* and *B. angulatum* inoculated at $6 \log_{10}$ CFU/mL into unfermented skim milk did not show significant loss of viability over the 15-day storage period under refrigerated conditions ($p < 0.05$). Similarly, Sanders and others (1996) reported that viability of *Bifidobacterium*

BG9 inoculated at $7 \log_{10}$ CFU/mL into 1.5% fat milk was not affected during a 21-day incubation period at 4°C .

Commercially available milk inoculated with *Bifidobacterium* and *L. acidophilus* (strains not specified) stored at $4 \pm 0.5^{\circ}\text{C}$ were studied by Shin and others (2000b). A significant reduction ($0.4 \log_{10}$ CFU/mL) in the *Bifidobacterium* population was observed in the commercial milk samples 3 days prior to the expiration date and the reduction increased to 71% ($0.5 \log_{10}$ CFU/mL) at the time of the product expiration. Even though the reduction was significant in this study, the log reduction observed was not greater than $1 \log_{10}$ CFU/mL until 6 days after the milk expired. Therefore, results from the study by Shin and others were considered similar to the results obtained in our study.

Survivability in simulated gastric juice

Being able to survive the acidity in the gastric juice produced in the human stomach is an important characteristic of *Bifidobacterium* to be used as probiotic dietary adjuncts. Several studies have been conducted in which the effect of the acid simulating gastric environment on the survivability of *Bifidobacterium* and other lactic acid bacteria were examined (Conway and others 1987; Berrada 1991; Clark and others 1993; Lankaputhra and Shah 1995; Charteris and others 1998; Lian and others 2003). *Bifidobacterium* strains used in this study survived the 4 hours of treatment with simulated pH 3.0 gastric juice prepared according to Lian and others (Figure 2 and Figure 6). This result agreed with the study conducted by Clark and others (1993). Clark and others observed that *B. infantis*, *B. longum*, *B. bifidum* and *B. adolescentis* in neomycin-paromomycin-nalidixic acid-lithium chloride (NPNL) broth survived pH 3.0 simulated gastric juice for 3 hours at 37°C . In addition to pH

3.0, they also showed that when treated with pH 2.0 simulated gastric juice, *B. infantis*, *B. longum* and *B. adolescentis* maintained their viability as well but *B. bifidum* lost their viability after one hour.

Results from study conducted by Lankaputhra and Shah (1995) were different than our study for some of the strains used. At pH 3.0, *B. bifidum* 1900 and 1901, *B. breve* 1930 and *B. longum* 20097 showed a rapid reduction in the viable counts over the 3 hours incubation period at 37°C, which contradicted the results observed in our study. However, survivability of *B. infantis* 1912 and *B. longum* 1941 were similar to the results observed in our study. The differences in the results observed might be due to the different methodology applied. An acidic environment was created by adjusting the pH of the culture broth with 5 N HCl in Lankaputhra and Shah's study, which is different than our study where 10 mL of the milk samples were added into 10 mL pH 3.0 gastric juice. Addition of milk into the gastric juice in our study increased the pH of the gastric juice to 6.56. With the pH close to neutral, the survivability of the *Bifidobacterium* was improved.

In addition to increasing the pH of the gastric juice, Charteris and others (1998) showed that with the addition of milk protein, the tolerance of *B. infantis* and *Lactobacillus casei* to simulated gastric juice increased to 100%. Similar results were observed by Conway and others (1987) with the addition of skim milk to lactic acid bacteria treated with human gastric juice. Charteris and others (1998) suggested that the buffering capacity of milk protein and the capability of milk protein to inhibit protease activity *in vivo* might help to protect the *Bifidobacterium* during upper gastrointestinal tract transit and thus increase their survivability.

From Figure 2, over the storage period, *Bifidobacterium* showed a slight improvement in viability after treatment with gastric juice for 4 hours. *Bifidobacterium* might have utilized the lactose in the milk and produced lactic acid. The production of the lactic acid decreased the pH of the milk and *Bifidobacterium* might have adapted to the lower pH gradually. Adaptation to the lower pH might have helped to improve their viability when exposed to the gastric juice.

Survivability in simulated bile solutions

To be used as dietary adjuncts, *Bifidobacterium* must survive through the bile concentrations produced in the human small intestine. Effects of bile solutions on *Bifidobacterium* during gastrointestinal tract transit were studied by several researchers (Rao and others 1989; Ibrahim and Bezkorovainy 1993; Clark and Martin 1994; Lankaputhra and Shah 1995; Charteris and others 1998; Lee and Heo 2000). Bile solutions used in our study were prepared according to Lian and others (2003) and Clark and Martin (1994). The concentration of bile solutions used was 1%. According to Ibrahim and Bezkorovainy (1993), normal bile concentrations in the human small intestine is 0.06%, and 2% is the maximal concentration found in the human small intestine during the first hour of digestion of food.

After treatment with 1% bile solutions for 4 hours at 37°C, log counts for the *Bifidobacterium* strains used in our study did not vary more than 1 log₁₀ CFU/mL (Figure 3 and Figure 7). Lankaputhra and Shah (1995) showed that when 1% bile salts were added into the reconstituted nonfat dry milk supplemented with glucose, yeast extract and L-cysteine hydrochloride (NGYC) broth containing *Bifidobacterium*, *B. infantis* 1912, *B. breve* 1930 and *B. longum* 1941 survived the treatment after 3 hours at 37°C, which agreed with the

results observed in our study. However, the results for *B. bifidum* conflicted with the results obtained in our study. The *B. bifidum* 1900 strains used in Lankaputhra and Shah's study showed a 1 log₁₀ CFU/mL reduction after 3 hours in their study and, rapid decline in the log count was observed in *B. bifidum* 1901 strains after 1 hour and a 4 log₁₀ CFU/mL reduction was observed after 3 hours.

Results from the study conducted by Clark and Martin (1994) were different than the results observed in our study. Clark and Martin showed that *B. infantis*, *B. adolescentis* and *B. bifidum* did not survive the bile solutions treatment and only *B. longum* used in their study survived bile solutions treatment. The differences observed are probably due to the different methodology applied. Clark and Martin incubated 1 mL *Bifidobacterium* containing broth into 10 mL of 2% bile solutions for 12 hours. The higher bile concentration used, the longer incubation time and the different amount of bile solutions applied in Clark and Martin's study might have contributed to the differences in the results.

Bifidobacterium's ability to survive the bile salts treatment might be due to the presence of bile salt hydrolase in the cell. Lepercq and others (2004) showed that living *Bifidobacteria animalis* DN-173 010 exhibited bile salt hydrolase activity in the gastrointestinal tract of the pig. With the presence of bile salt hydrolase, bile salts in the simulated bile solutions used in our study were hydrolyzed. Therefore, without the action of the bile salts in the solutions, *Bifidobacterium* were able to survive the 4 hours simulated bile solutions treatment.

Lactose Content

The presence of lactase activity in *B. bifidum* R0071 milk observed in this study was similar to that in the study conducted by Passerat and Desmaison (1995), which showed that lactase activity, was observed in *B. bifidum* fermented milk. *B. bifidum* fermented milk was added into 20 mL of 0.1 M phosphate buffer and adjusted to pH 6.5. The samples were incubated at 40°C, and, after 2 hours, lactose content was reduced from 2.96 g/100g to 1.35 g/100g.

A study conducted by Jiang and Savaiano (1997) demonstrated lactase activity in *B. longum* B6 in an *in vitro* fermentation process simulating ileostomy effluent containing 3.5% lactose. The results observed in Jiang and Savaiano disagreed with the results from our study. The difference in the results can be explained by the different growth conditions used in the studies. The *B. longum* R0175 used in this study was grown in MRS broth with 0.05% cysteine supplement. However, the *B. longum* B6 used in Jiang and Savaiano's study was grown in MRS broth supplemented with lactose as the sole carbohydrate source to adapt *B. longum* B6 to utilize lactose and express lactase activity. Therefore, lactase activity was observed during the fermentation process.

The adaptation of *B. longum* B6 to express lactase activity is further demonstrated in another study conducted by Jiang and others (1996). Jiang and others showed that when *B. longum* B6 was grown with lactose as the sole carbohydrate source, the β -galactosidase activity was higher (1.411 U/mL) compared to the *B. longum* B6 that was grown with both lactose and glucose (0.074 U/mL) as carbohydrate sources. They concluded that when *B. longum* is grown in medium containing only lactose as the sole carbohydrate source, a higher lactase activity is induced.

Effect of Supplements on the Survivability of *B. bifidum* R0071 in Milk

To increase the viability of *Bifidobacterium* in a food system, supplements that act as *Bifidobacterium* growth promoters can be added. Whey protein concentrate (WPC) and fructooligosaccharides (FOS) are widely used in the dairy industry to enhance the growth of *Bifidobacterium* in dairy products. Studies with 2% and 3% WPC or FOS supplementation have been shown to improve the survivability of *Bifidobacterium* in yogurt (Dave and Shah 1998; Shin and others 2000a; Bruno and others 2002; Martín–Diana and others 2003; Akalin and others 2004; Janer and others 2004). Even though many studies have focused on the supplement effects on the viability of *Bifidobacterium* in food products, no published studies have examined the effects of WPC and FOS on the survivability of *Bifidobacterium* during gastrointestinal tract transit.

Whey protein concentrate

In our study, over the storage period, treatments with WPC supplement had a similar survival rate compared to the control treatment. However, treatments with FOS supplement showed a reduction in log count over the storage period at 4°C (Figure 8). The similar survivability observed in the WPC supplemented samples to the control was desirable and was similar to the study conducted by Dave and Shah (1998) with yogurt. Dave and Shah supplemented whey protein concentrate into yogurt and the survivability of *Bifidobacterium* in the yogurt samples were maintained at 6 log₁₀ CFU/mL over the 35 days storage period at 4°C.

Janer and others (2004) observed a significantly better growth, 2 log₁₀ CFU/mL, when skim milk is supplemented with WPC and incubated at 37°C for 24 hours compared to

the control with no supplements. However, no significant growth was observed in our study when *B. bifidum* R0071 milk samples with WPC supplement were stored at 4°C for 15 days. This can be explained by the different incubation temperature used in our study. The incubation temperature, 4°C, is not the optimum growth temperature for *Bifidobacterium*, thus, no growth was observed in our samples.

Fructooligosaccharides

The decrease in viability observed in FOS supplemented treatments were different than the results obtained by Akalin and others (2004). Akalin and others supplemented yogurt with FOS and the viability of the *Bifidobacterium* increased over the 28-day storage time at 4°C compared to the control samples without FOS. Shin and others (2000) also showed that with the addition of FOS, growth of *Bifidobacterium* was enhanced in the skim milk when incubated at 37°C for 48 hours, and increased viability was observed when stored at 4°C for four weeks.

The reduction in the microbial counts during the storage period might be attributed to the utilization of FOS by *B. bifidum* R0071. Bielecka and others (2002) showed that *Bifidobacterium* has a higher growth rate when grown in FOS and oligofructose medium compared to the control medium with lactose as the main carbohydrate source. As *Bifidobacterium* grows, production of lactic acid and acetic acid from the fermentation of FOS might have inhibited or reduced the viability of *Bifidobacterium* itself over the storage period and thus contributed to the lower counts in the FOS-supplemented samples.

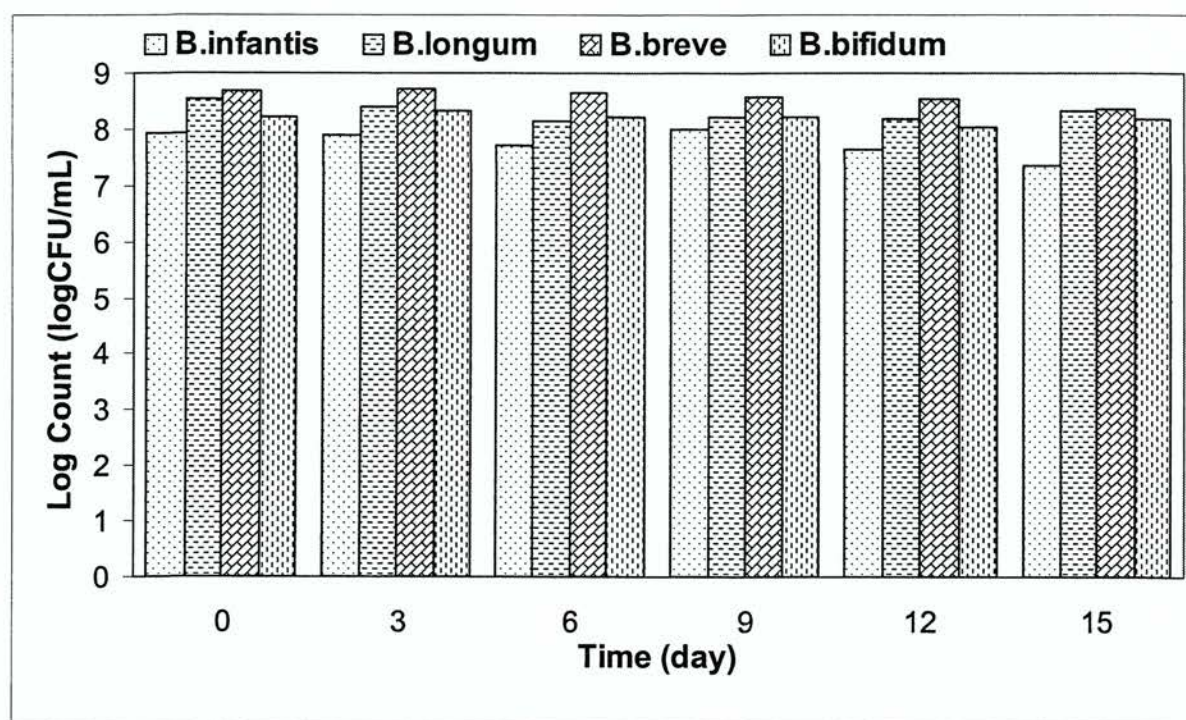


Figure 1 Viability of *Bifidobacterium* strains in milk stored for 15 days at 4°C

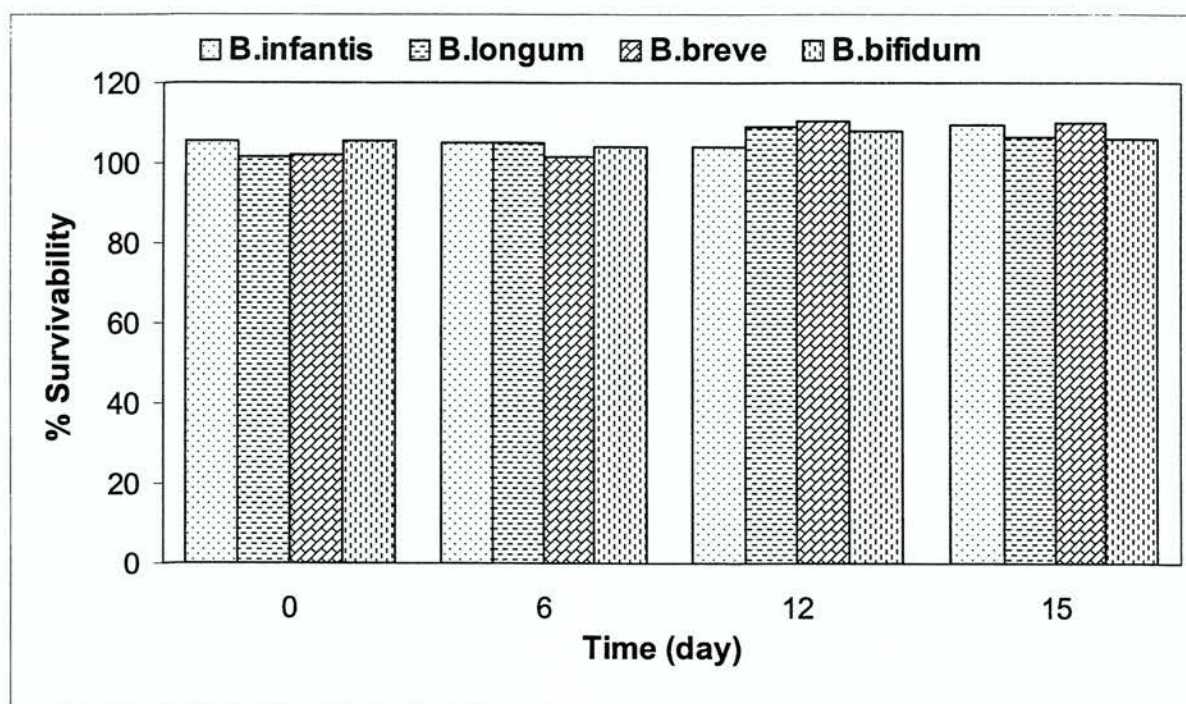


Figure 2 Percent survivability of *Bifidobacterium* strains in milk stored for 15 days at 4°C after 4 hours simulated gastric juice treatment

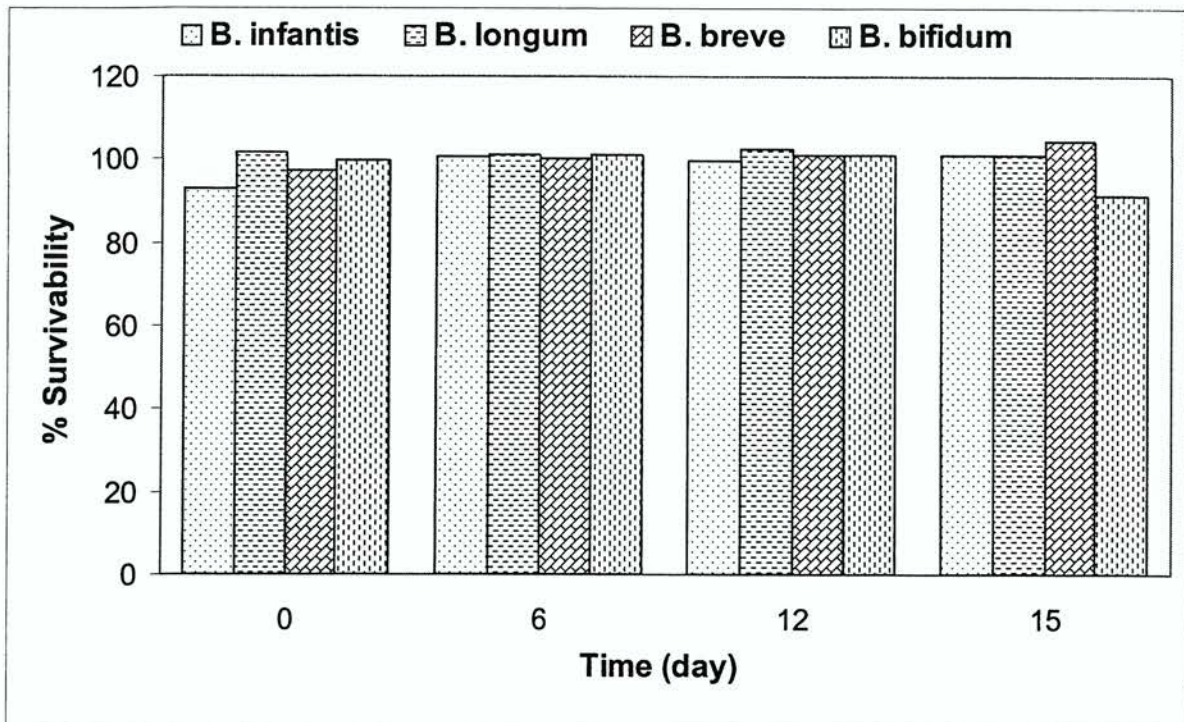


Figure 3 Percent survivability of *Bifidobacterium* strains in milk stored for 15 days at 4°C after 4 hours simulated bile solutions treatment

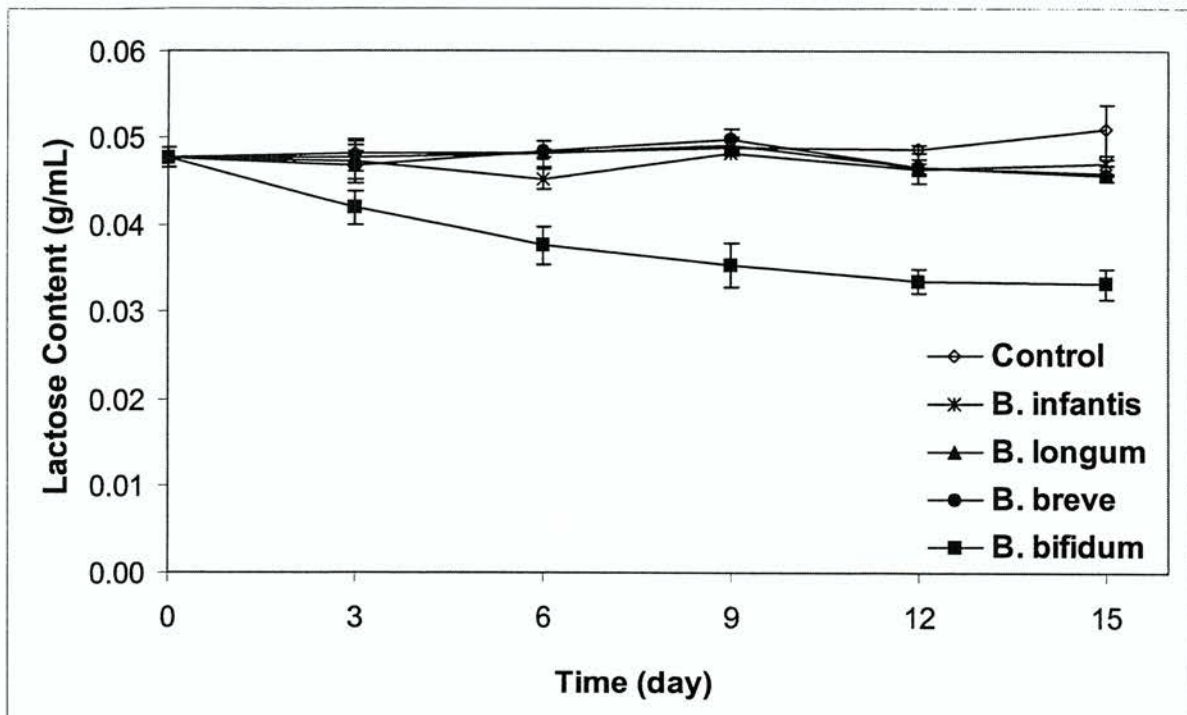


Figure 4 Lactose content in milk samples inoculated with *Bifidobacterium* strains during the 15-day storage time

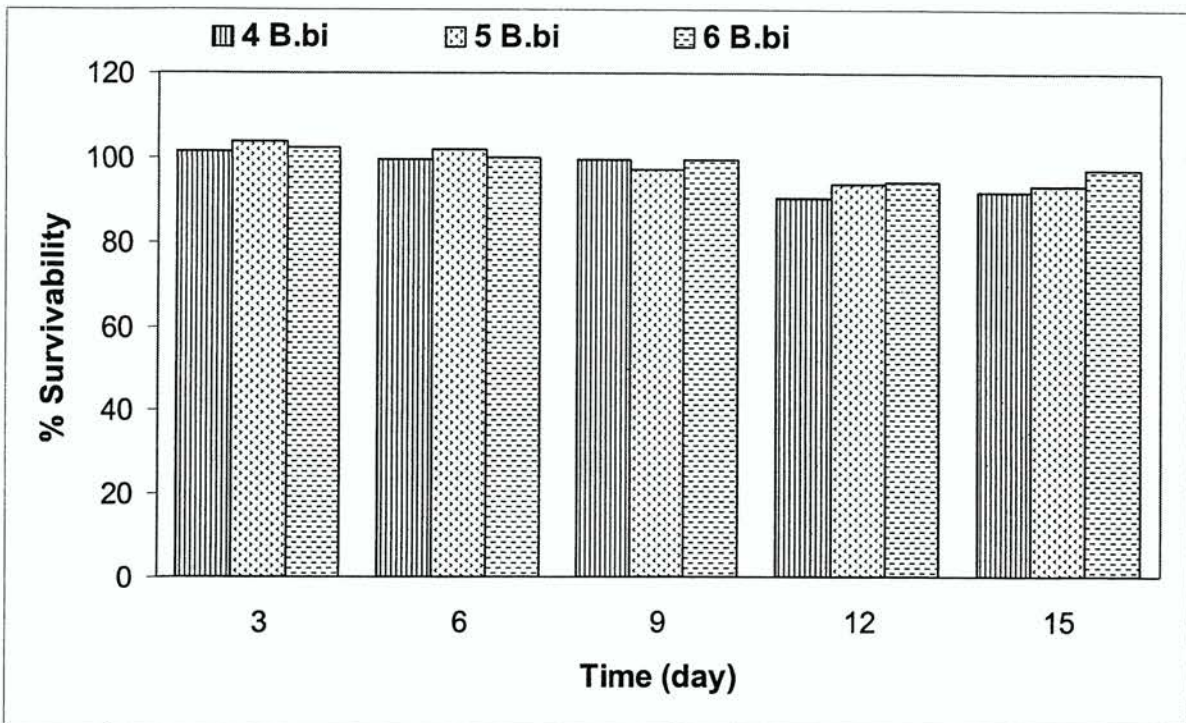


Figure 5 Percent survivability relative to day 0 of *Bifidobacterium bifidum* R0071 inoculated into milk at 3 different concentrations and stored for 15 days at 4°C

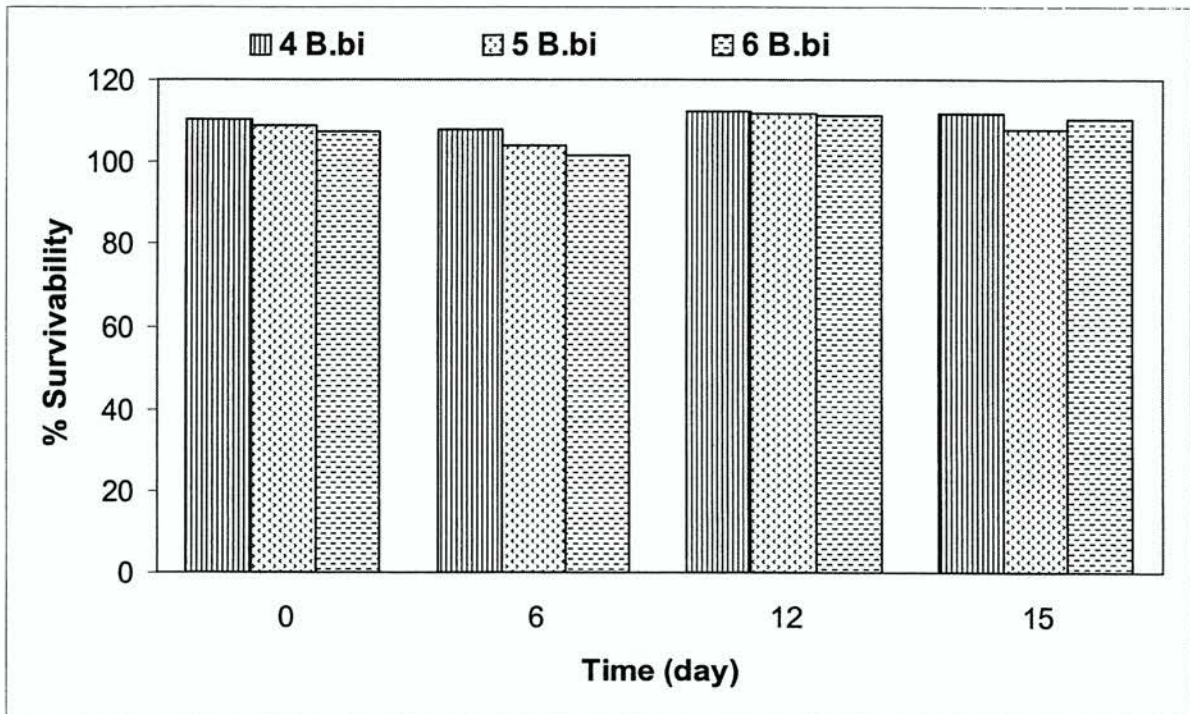


Figure 6 Percent survivability of *B. bifidum* R0071 inoculated into milk at 3 different concentrations and stored for 15 days at 4°C after 4 hours simulated gastric juice treatment

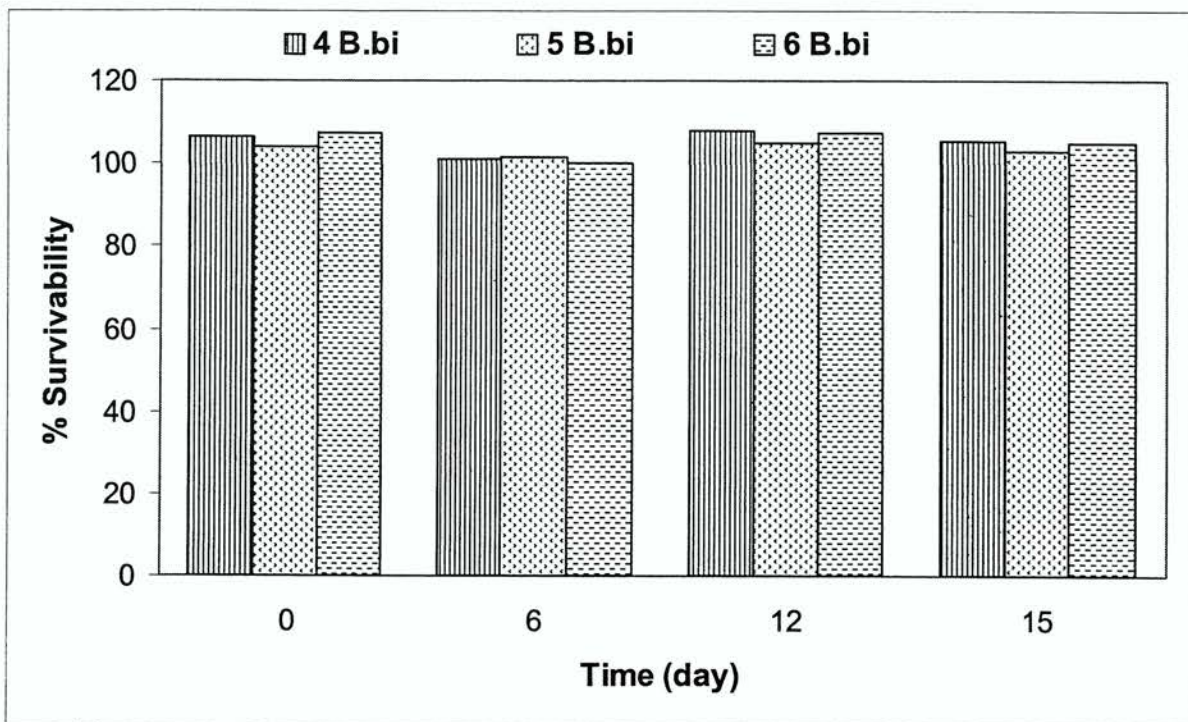


Figure 7 Percent survivability of *B. bifidum* R0071 inoculated into milk at 3 different concentrations and stored for 15 days at 4°C after 4 hours simulated bile solutions treatment

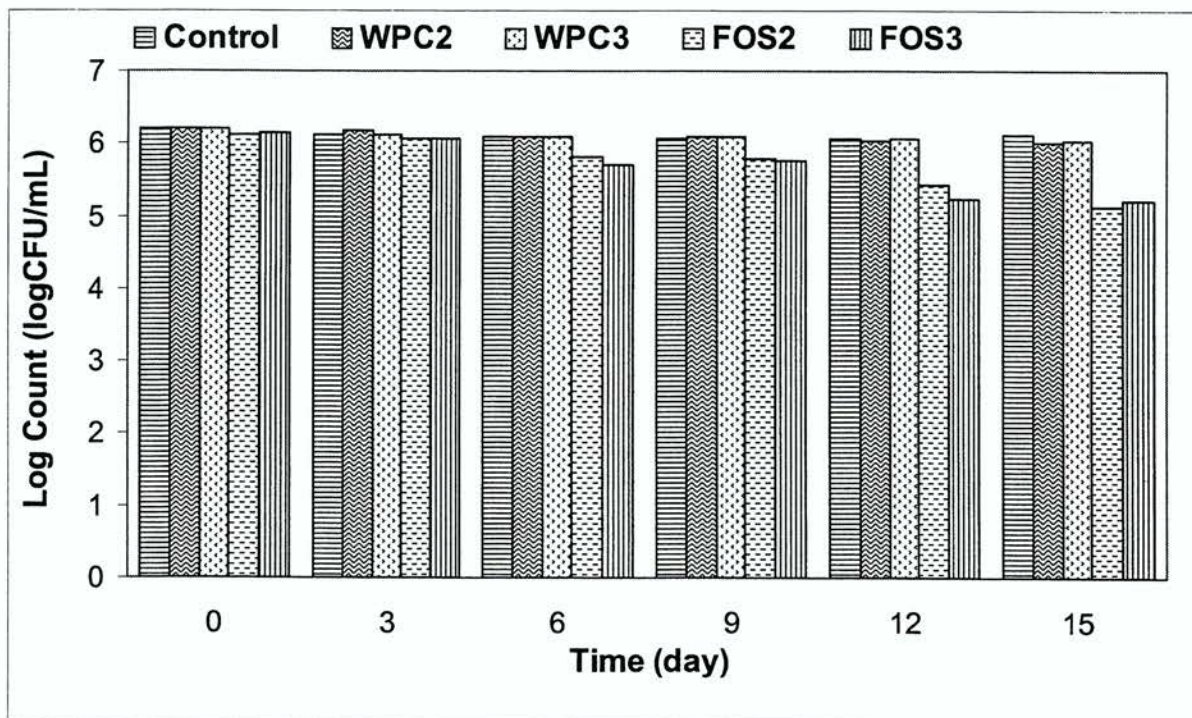


Figure 8 Viability of *B. bifidum* R0071 with added supplements in milk during 15 days storage period at 4°C

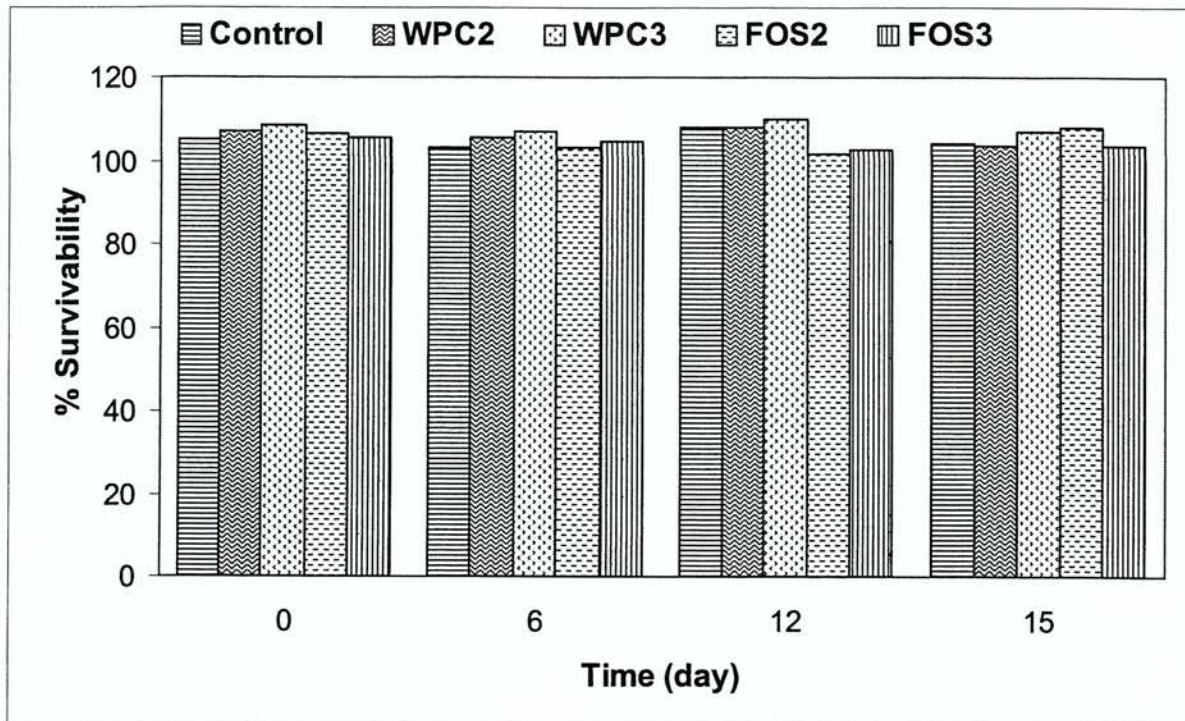


Figure 9 Percent survivability of *B. bifidum* R0071 inoculated into milk with supplements and stored for 15 days at 4°C after 4 hours simulated gastric juice treatment

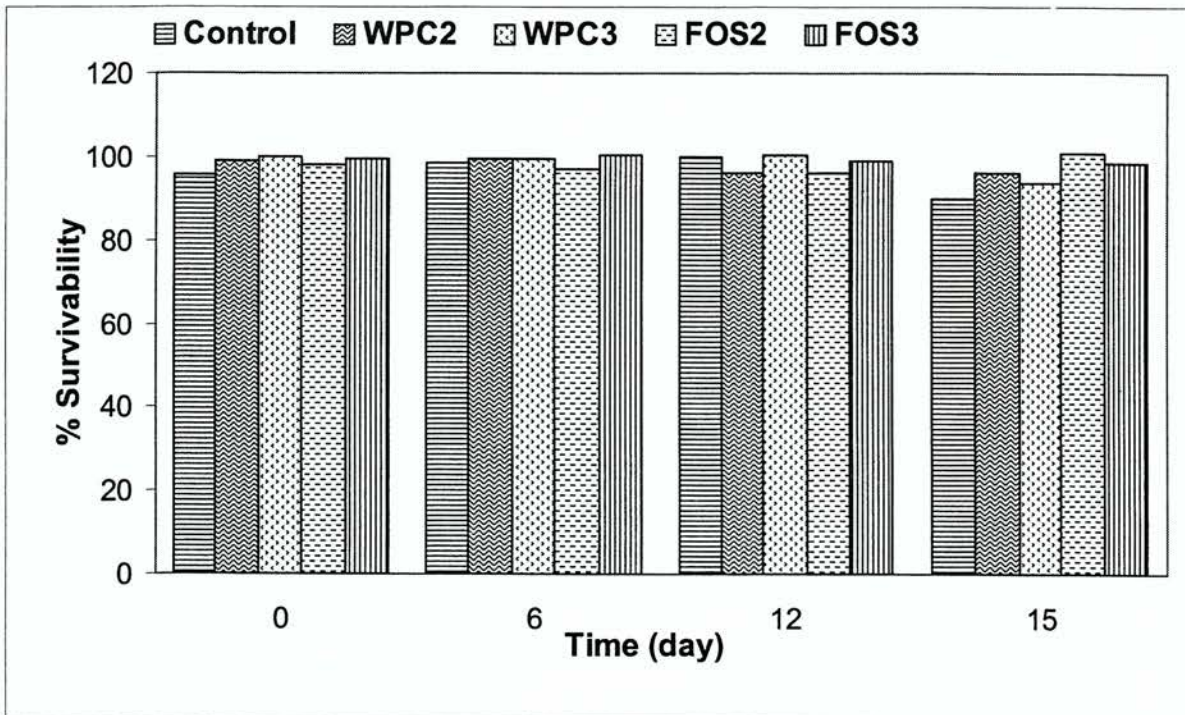


Figure 10 Percent survivability of *B. bifidum* R0071 inoculated into milk with supplements and stored for 15 days at 4°C after 4 hours simulated gastric juice treatment

CONCLUSION

The *Bifidobacterium* strains, *B. bifidum* R0071, *B. breve* R0070, *B. infantis* R0033 and *B. longum* R0175 used in this study survived over the 15-day storage period. *B. bifidum* R0071 is the only *Bifidobacterium* strain used in this study that hydrolyzed lactose. Different inoculation level did not affect the viability of *B. bifidum* R0071 significantly over the 15-day storage study. Whey protein concentrate (WPC) and fructooligosaccharides (FOS) supplements used in this study did not increase the viability and growth of the *B. bifidum* R0071 significantly compared with those of the control.

The changes in the log count during the storage period and after the treatment with simulated gastric juice and bile solutions were not greater than 1 log₁₀ CFU/mL in all 3 studies, which showed that milk, can be used as an effective carrier for the delivery of *Bifidobacterium* into the human gut. Inoculation of *Bifidobacterium* at 6 log₁₀ CFU/mL into milk without WPC and FOS supplement is recommended. A serving size for milk is approximately 236 mL, and, at this inoculation level, consumer would be able to obtain approximately 8 log₁₀ CFU, which is the recommended therapeutic dose, in 1 serving of milk.

Simulated gastrointestinal tract transit used in this study was not performed under anaerobic conditions, thus, oxygen stress might affect the viability of *Bifidobacterium* during the treatment. In addition, the amount of simulated gastric juice and bile solutions used to treat the milk samples in this study were conducted with 1:1 ratio, which might not be the accurate amount of gastric juice and bile solutions secreted in human gastrointestinal tract. Therefore, a different survivability might be observed when *Bifidobacterium* milk samples were consumed in human clinical trials.

Sequential application of simulated gastric juice and bile solutions, which is more comparable to the human gastrointestinal tract transit, can be conducted. The survivability of *Bifidobacterium* in the simulated bile solutions might be affected after treatment with simulated gastric juice. Clinical studies with human subjects can be conducted as well to compare the results from the simulated gastrointestinal tract transit performed in this experiment to the results from the *in vivo* study. Clinical studies can further provide information to researchers regarding the survivability of *Bifidobacterium* during gastrointestinal tract transit.

During the simulated gastrointestinal tract transit, samples were incubated at 37°C, which is the optimal growth temperature for *Bifidobacterium*. Incubation at this temperature might stimulate the growth of *Bifidobacterium* in the samples and lactose might be utilized by *Bifidobacterium* as the energy source. Therefore, lactose measurement can be conducted after the 4-hour simulated gastrointestinal tract transit to obtain an estimation of an *in vitro* lactose utilization rate during the gastrointestinal tract transit.

Other supplements that were studied and presented positive effects, such as lactulose, inulin, acid casein hydrolysate, tryptone and caseinomacropeptide, can be studied to examine their effects on enhancing the survivability and growth of *Bifidobacterium* in the milk over the storage period and during the simulated gastrointestinal tract transit (Modler and others 1990; Gibson and others 1995; Dave and Shah 1998). Effects of a combination of different supplements on the survivability of *Bifidobacterium* can be conducted to obtain a good and effective combination of supplements as well.

Sensory quality of the food is important. With the incorporation of *Bifidobacterium* into the milk, the sensory properties of the milk might be affected. Addition of supplements

into the milk can also affect the sensory properties of the milk as well. Therefore, sensory studies should be conducted to examine the effects of the *Bifidobacterium* and the supplements on the aroma and flavor of the milk.

Bifidobacterium survived in the milk throughout the shelf life of the milk and after the simulated gastrointestinal tract transit treatment. Therefore, milk can be used as a carrier medium to deliver *Bifidobacterium* and their therapeutic health benefits to the society.

APPENDIX A

Effects of Supplements on the Survivability of *Bifidobacteria longum* in Milk

B. longum R0175 was inoculated into milk samples at 10^9 CFU/mL and stored at 4°C for 15 days. Ascorbic acid, cysteine or ascorbic acid plus cysteine were added into *B. longum* R0175 milk samples at 0.05% concentration. Microbial analysis and lactose utilization were conducted at 3 day intervals for 15 days. Survivability during gastrointestinal tract transit was conducted with pH 2.0 gastric juice and 1% bile solutions at day 0, 6, 12 and 15. *B. longum* R0175 milk samples (1 mL) were added into 9 mL gastric juice or 9 mL bile solutions and incubated in 37°C water bath for 4 hours. Samples were plated on MRSC agar and incubated anaerobically at 37°C for 72 hours.

Over the 15-day storage time, the survivability of *B. longum* R0175 was maintained at over 8 log₁₀ CFU/mL (Figure 1). Supplements with cysteine and ascorbic acid did not show any effect on the growth or reduction of *B. longum* R0175 in milk over the storage time. When treated with gastric juice and bile solutions, reduction in log counts was observed in all samples. After 4 hours of treatment with pH 2.0 gastric juice, *B. longum* R0175 in all milk samples except cysteine supplemented samples, did not survive, as shown by counts of less than 1 log₁₀ CFU/mL (data not shown). Cysteine supplemented *B. longum* R0175 milk samples survived the gastric juice treatment and at day 15, counts of *B. longum* R0175 were at 5.13 log₁₀ CFU/mL. Bile solutions treatment affected the viability of *B. longum* R0175 in milk. At day 15, all milk samples had less than 1 log₁₀ CFU/mL after treatment with 1% bile solutions for 4 hours (data not shown).

Samples supplemented with cysteine showed a reduction in lactose content at day 9, and at day 15, 53% reduction in lactose content was observed (Figure 2). *B. longum* R0175 milk samples have a lower lactose content compared to the control milk samples with no *B. longum* R0175 inoculated over the storage time.

Sensory properties of the milk samples were examined at each sampling day by sniffing the aroma of the milk. Undesirable sulfur odor was observed for the milk samples supplemented with cysteine. Samples supplemented with ascorbic acid have a stronger sour aroma compared to the control sample with no supplement over the storage time.

Supplements did not enhance the viability of *B. longum* R0175 in milk samples over the storage time. Cysteine supplement enhanced the viability of *B. longum* R0175 when treated with pH 2.0 gastric juice. However, due to the undesirable aroma that affected the sensory property of the milk, we did not proceed with this supplement. In addition, curdling was observed in all samples after 15 days of storage. Therefore, a lower inoculation level is preferred to preserve the sensory quality of the milk.

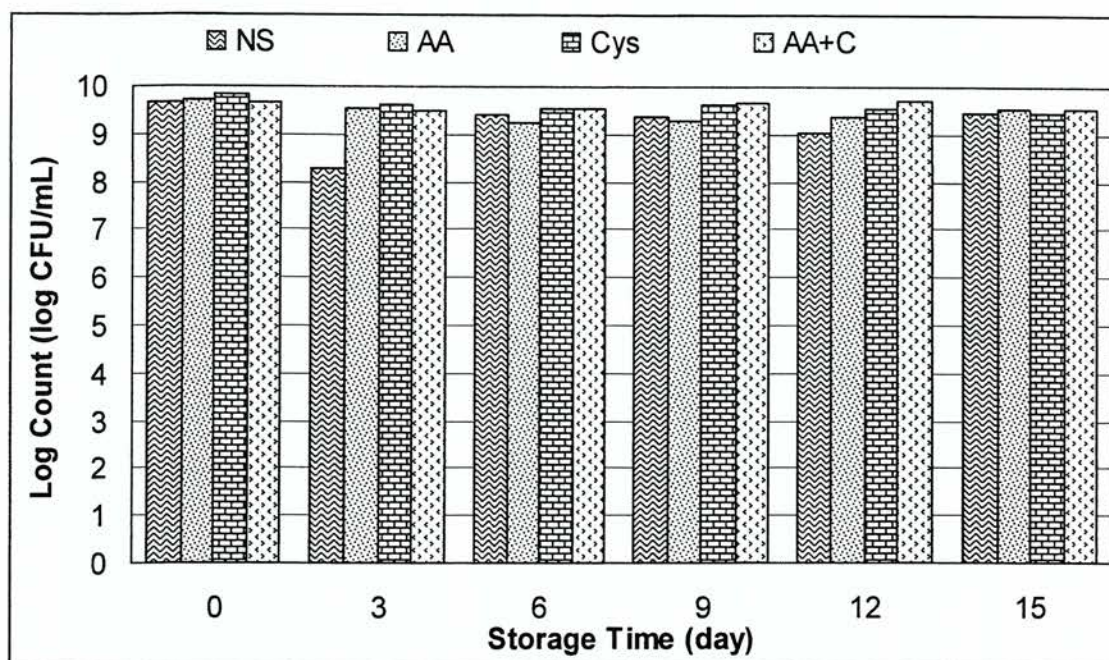


Figure 1 Survivability of *Bifidobacteria longum* R0175 with added supplements^a in milk stored for 15 days at 4°C

^aNS = No supplement, AA = Ascorbic acid supplement, Cys = Cysteine supplement, AA+C = Ascorbic acid and cysteine supplements

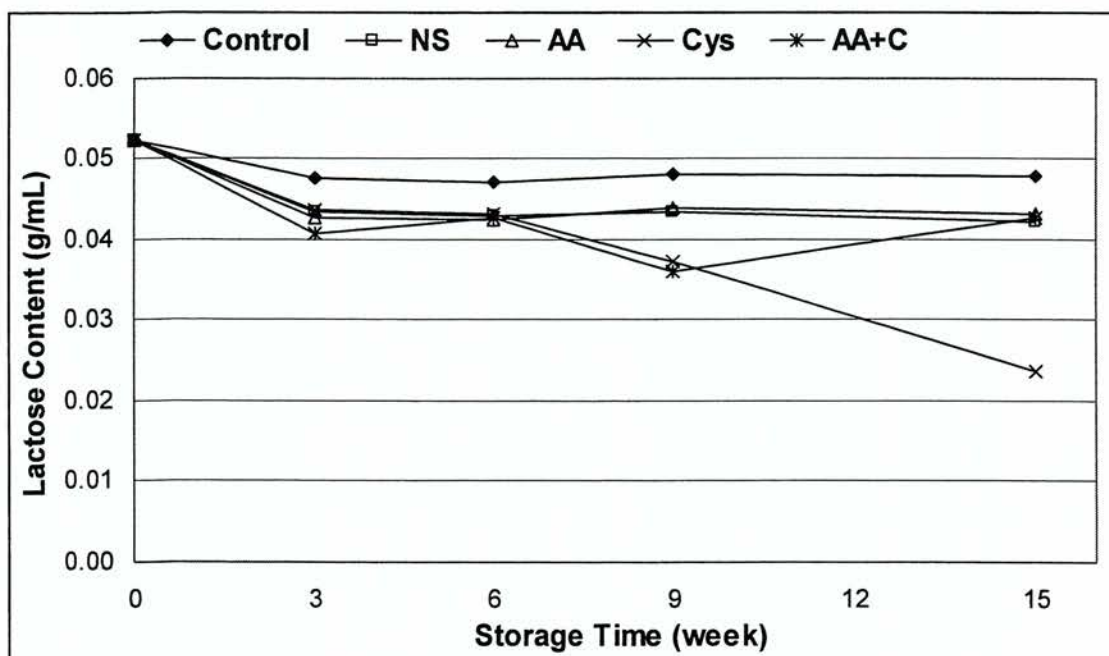


Figure 2 Lactose content in milk samples inoculated with *Bifidobacteria longum* R0175 with added supplements^a during the 15 days storage time.

^aControl = Uninoculated Milk NS = No supplement, AA = Ascorbic acid supplement, Cys = Cysteine supplement, AA+C = Ascorbic acid and cysteine supplements

APPENDIX B

Quality of Milk Packaged in Polylactic Acid and Polyethylene Terephthalate Bottles

Environmental concerns related to the use of plastic materials for food containers have contributed to the development of packaging materials from renewable resources that are biodegradable. Polylactic acid (PLA) is a plastic made from corn sugars and is cost competitive with current petroleum-based plastics. Although PET (polyethylene terephthalate) bottles are widely used for food packaging, these bottles are non-biodegradable. Food-package interactions can influence the nutritional, flavor, and microbial quality of the food products. Light penetration can also have a significant impact on sensory and nutritional quality through the degradation of riboflavin and formation of light-struck flavor.

The objective of this study was to compare the microbiological, sensory, and nutritive quality of milk packaged in PLA and PET bottles. Moisture migration in PLA and PET bottles were examined as well.

Milk was packaged in PLA or PET bottles. Milk samples stored at 4 and 10 °C were analyzed using standard plate count methods to monitor microbiological quality. Milk samples were stored in light or dark (control) for 4 hours to determine effects of light exposure and packaging material and sensory quality and riboflavin content. Sensory quality was evaluated using triangle tests to compare all combinations of light exposure and packaging treatment. Moisture migration was conducted by filling PLA and PET bottles with 500g of water and stored at 4 and 10°C for 5 weeks.

Storage time, but not packaging material had a significant effect on microbial growth. Microbial counts exceeded 10^7 CFU/mL after 14 and 21 days, when stored at 10 °C and 4 °C,

respectively (Figure 1). As shown in Table 1, of the light exposure and packaging treatment combinations evaluated, sensory panelists were only able to detect differences in light-exposed and control milk packaged in PET bottles. Table 2 showed that light exposure reduced riboflavin content from 0.19 to 0.16 mg/100 mL milk. However, packaging material did not affect riboflavin content. PLA bottles showed a higher loss of weight compare to PET bottles when stored at 10°C. After 5 weeks storage, PLA bottles lost 5.73 g and 0.90 g, when stored at 10°C and 4°C, and PET bottles lost 0.00g and 0.47 g, respectively (Figure 2).

Comparison of PLA and PET bottles for milk resulted in no differences in microbiological, sensory, or nutritive quality. PLA is an environmentally friendly alternative to PET for packaging milk.

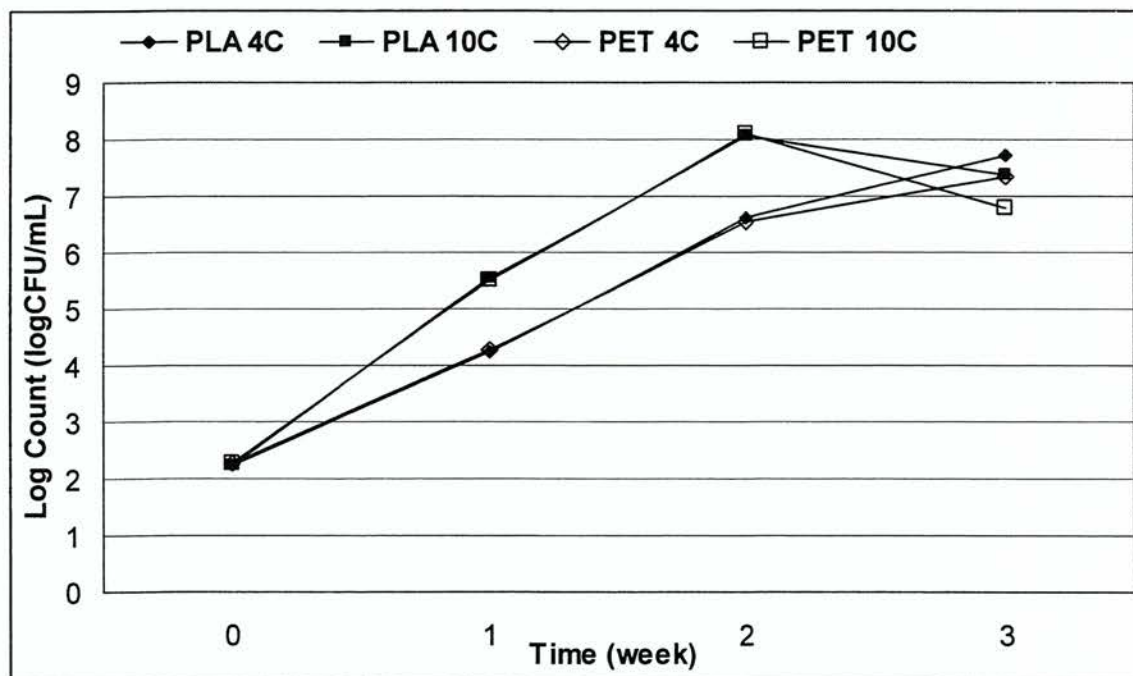


Figure 1 Effect of packaging material^a and storage temperature^b on total aerobic plate count

^a PLA = Polylactic acid, PET = Polyethylene terephthalate

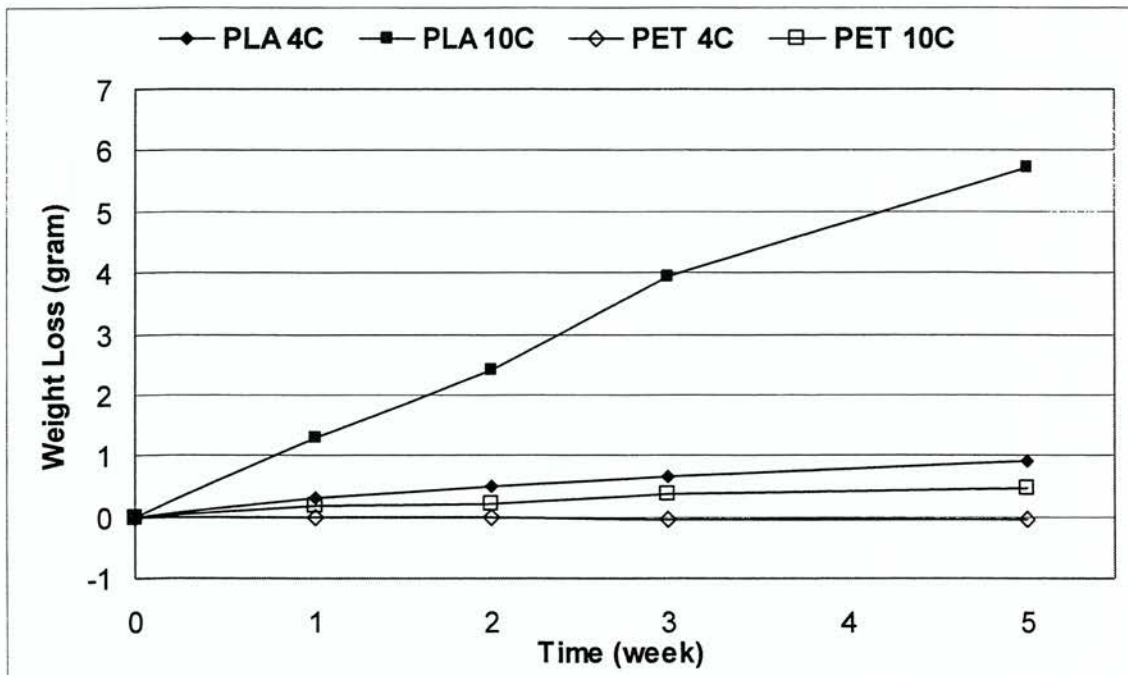
^b 4C = 4°C, 10C = 10°C

Table 1 Effect of packaging material and light exposure on sensory quality of milk

<i>Constant Factor</i>	<i>Treatment Variable</i>	<i>Differences detected by Panelists ($\alpha < 0.05$)</i>
PET Bottles	Light vs Dark	Yes
PLA Bottles	Light vs Dark	No
Light Exposure	PLA vs PET	No
Dark	PLA vs PET	No

Table 2 Effect of packaging material and light exposure on riboflavin content^a of milk

Packaging Material	<i>Treatment</i>		Average
	Light	Dark	
PLA	0.15	0.20	0.18
PET	0.16	0.18	0.17
Average	0.16	0.19	

^a mg/100 mL milk**Figure 2** Weight loss from PLA and PET bottles filled with water during storage at 4 and 10°C

REFERENCES

- Akalin AS, Fenderya S, Akbulut N. 2004. Viability and activity of *Bifidobacteria* in yoghurt containing fructooligosaccharide during refrigerated storage. *Int J Food Sci Tech* 39:613–21.
- Asahara T, Nomoto K, Shimizu K, Watanuki M, Tanaka R. 2001. Increased resistance of mice to *Salmonella enterica* serovar *Typhimurium* infection by symbiotic administration of *Bifidobacteria* and transgalactosylated oligosaccharides. *J Appl Microbiol* 91:985–96.
- Asahara T, Shimizu K, Nomoto K, Hamabata T, Ozawa A, Takeda Y. 2004. Probiotic *Bifidobacteria* protects mice from lethal infection with shiga toxin-producing *Escherichia coli* O157:H7. *Infect Immun* 72:2240–7.
- Barbut F, Richard A, Hamadi K, Chomette V, Burghoffer B, Petit JC. 2000. Epidemiology of recurrences or reinfections of *Clostridium difficile*-associated diarrhea. *J Clin Microbiol* 38:2386–8.
- Bartlett JG. 2002. Antibiotic-associated diarrhea. *N Eng J Med* 346:334–9.
- Beaugerie L, Petit JC. 2004. Antibiotic-associated diarrhoea. *Best Pract Res Clin Gastroenterol* 18:337–52.
- Bengmark S. 1996. Ecnutrition and health maintenance—A new concept to prevent GI inflammation, ulceration and sepsis. *Clin Nutr* 15:1–10.
- Bergogne-Bérézin E. 2000. Treatment and prevention of antibiotic associated diarrhea. *Int J Antimicrob Agents* 16:521–6.

- Bergonzelli GE, Blum S, Brüssow H and Corthésy-Theulaz I. 2005. Probiotics as a treatment strategy for gastrointestinal diseases? *Digestion* 72:57–68.
- Bernet MF, Brassart D, Neeser JR, Servin AL. 1993. Adhesion of human *bifidobacterial* strains to cultured human epithelial cells and inhibition of enteropathogen-cell interactions. *Appl Environ Microbiol* 59:4121–8.
- Berrada N, Lemeland JF, Laroche G, Thouvenot P, Piaia M. 1991. *Bifidobacterium* from fermented milks: Survival during gastric transit. *J Dairy Sci* 74:409–13.
- Bezkorovainy A. 2001. Probiotics: determinants of survival and growth in the gut. *Am J Clin Nutr* 73:399S–405S.
- Biavati B, Vescovo M, Torriani S, Bottazzi V. 2000. *Bifidobacteria*: history, ecology, physiology and applications. *Ann Microbiol* 50:117–31.
- Bielecka M, Biedrzycka E, Majkowska A. 2002. Selection of probiotics and probiotics for synbiotics and confirmation of their *in vivo* effectiveness. *Food Res Int* 35:125–31.
- Boehm G, Lidestri M, Casetta P, Jelinek J, Negretti F, Stahl B, Marini A. 2005. Supplementation of a bovine milk formula with an oligosaccharide mixture increases counts of faecal *bifidobacteria* in preterm infants. *Arch Dis Child Fetal Neonatal Ed* 86:F178–81.
- Bouhnik Y, Pochart P, Marteau P, Arlet G, Goderel I, Rambaud JC. 1992. Fecal recovery in humans of viable *Bifidobacterium* species ingested in fermented milk. *Gastroenterology* 102: 875–8.
- Boyle P, Langman JS. 2000. ABC of colorectal cancer. *Epidemiology. Br Med J* 321:805–8.
- Brady LJ, Gallaher DD, Busta FF. 2000. The role of probiotic cultures in the prevention of colon cancer. *J Nutr* 130:410S–4S.

- Bruno EA, Lankaputhra WEV, Shah NP. 2002. Growth, viability and activity of *Bifidobacterium* spp. in skim milk containing probiotics. J Food Sci 67:2740–4.
- Buchanan RE, Gibbon NE. 1974. Bergey's manual of determinative bacteriology. 8th ed. Baltimore: The Williams & Wilkins Company. p 1268.
- Bury D, Jelen P, Kimura K. 1998. Whey protein concentrate as a nutrient supplement for lactic acid bacteria. Int Dairy J 8:149–51.
- Casburn–Jones AC, Farthing MJG. 2004. Traveler's diarrhea. J Gastroenterol Hepatol 19:610–8.
- Chandan RC. 1999. Enhancing market value of milk by adding cultures. J Dairy Sci 82:2245–56.
- Charteris WP, Kelly PM, Morelli L, Collins JK. 1998. Development and application of an in vitro methodology to determine transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. J Appl Microbiol 84:759–68.
- Chung HS, Kim YB, Chun SL, Ji GE. 1999. Screening and selection of acid and bile resistant *bifidobacteria*. Int J Food Microbiol 47:25–32.
- Clark PA, Cotton LN, Martin JH. 1993. Selection of *Bifidobacteria* for use as dietary adjuncts in cultured dairy foods: II–Tolerance to simulated pH of human stomachs. Cult Dairy Prod J 28:11–14.
- Clark PA, Martin JH. 1994. Selection of *Bifidobacteria* for use as dietary adjuncts in cultured dairy foods: III–Tolerance to simulated bile concentrations of human small intestines. Cult Dairy Prod J 29:18–21.

- Collins MD, Gibson GR. 1999. Probiotics, prebiotics and synbiotics: approaches for modulating the microbial ecology of the gut. *Am J Clin Nutr* 69:1052S–7S.
- Colombel JF, Cortot A, Neut C, Romond C. 1987. Yoghurt with *Bifidobacterium longum* reduces erythromycin-induced gastrointestinal effects. *Lancet* 330(8549):43.
- Commane D, Hughes R, Shortt C, Rowland I. 2005. The potential mechanisms involved in the anti-carcinogenic action of probiotics. *Mutat Res* 591:276–89.
- Conway PL, Gorbach SL, Goldin BR. 1987. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. *J Dairy Sci* 70:1–12.
- Dave RI, Shah NP. 1997a. Effectiveness of ascorbic acid as an oxygen scavenger in improving viability of probiotic bacteria in yoghurts made with commercial starter cultures. *Int Dairy J* 7:435–43.
- Dave RI, Shah NP. 1997b. Effect of cysteine on the viability of yoghurt and probiotic bacteria in yoghurts made with commercial starter cultures. *Int Dairy J* 7:537–45.
- Dave RI, Shah NP. 1998. Ingredient supplementation effects on viability of probiotic bacteria in yogurt. *J Dairy Sci* 81:2804–16.
- De Roos NM, Katan MB. 2000. Effects of probiotic bacteria on diarrhoea, lipid metabolism, and carcinogenesis: A review of papers published between 1988 and 1998. *Am J Clin Nutr* 71:405–11.
- De Roos NM, Schouten G, Katan MB. 1999. Yoghurt enriched with *Lactobacillus acidophilus* does not lower blood lipids in healthy men and women with normal to borderline high serum cholesterol levels. *Eur J Clin Nutr* 53:277–80.
- De Smet I, De Boever P, Verstraete W. 1998. Cholesterol lowering in pigs through enhanced bacterial bile salt hydrolase activity. *Br J Nutr* 79:185–94.

- De Smet I, Van Hoorde L, De Saeyer N, Vande Woestyne M, Verstraete W. 1994. *In vitro* study of bile salt hydrolase (BSH) activity of BSH isogenic *Lactobacillus plantarum* 80 strains and estimation of cholesterol lowering through enhanced BSH activity. *Microbial Ecol Health Dis* 7:315–29.
- De Vrese M, Stegelmann A, Richter B, Fenselau S, Laue C, Schrezenmeir J. 2001. Probiotics—compensation for lactase insufficiency. *Am J Clin Nutr* 73:421S–9S.
- Dong X, Xin Y, Jian W, Liu X, Ling D. 2000 *Bifidobacterium thermacidophilum* sp. nov., isolated from an anaerobic digester. *Int J Syst Evol Microbiol* 50:119–25.
- Dunne C, Murphy L, Flynn S, O'Mahony L, O'Halloran S, Feeney M, Morrissey D, Thornton G, Fitzgerald G, Daly C, Kiely B, Quigley EMM, O'Sullivan GC, Shanahan F, Collins JK. 1999. Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. *Antonie Van Leeuwenhoek* 76:279–92.
- Dunne C, O'Mahony L, Murphy L, Thornton G, Morrissey D, O'Halloran S, Feeney M, Flynn S, Fitzgerald G, Daly C, Kiely B, O'Sullivan GC, Shanahan F, Collins JK. 2001. *In vitro* selection criteria for probiotic bacteria of human origin: correlation with *in vivo* findings. *Am J Clin Nutr* 73:386S–92S.
- DuPont HL, Ericsson CD. 1993. Prevention and treatment of traveler's diarrhea. *N Engl J Med* 328:1821–7.
- DuPont HL. 1997. *Lactobacillus* GG in prevention of traveler's diarrhea: an encouraging first step. *J Travel Med* 4:1–2.
- Ericsson CD. 2003. Travellers' diarrhoea. *Int J Antimicrob Agents* 21:116–24.

[FAO/WHO] United Nation World Health Organization/Food and Agriculture Organization.

2001. Report of a Joint FAO/WHO Expert Consultation. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria.

Available from:

http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf.

Accessed Jan 27, 2006.

Feagan B. 1997. Antibiotics are not effective therapy for Crohn's disease (Time to remove the rose-coloured glasses). *Inflamm Bowel Dis* 3:314–7.

Fooks LJ, Gibson GR. 2002. Probiotics and modulators of the gut flora. *Br J Nutr* 88:S39–S49.

Fuller R. 1992. History and development of probiotics. In: Fuller R, editor. *Probiotics: The scientific basis*. London: Chapman & Hall. p 1–8.

Gagnon M, Kheadr EE, Le Blay G, Fliss I. 2004. *In vitro* inhibition of *Escherichia coli* O157:H7 by *bifidobacterial* strains of human origin. *Int J Food Microbiol* 92:69–78.

Gibson GR, Beatty ER, Wang X, Cummings JH. 1995. Selective stimulation of *Bifidobacteria* in the human colon by oligofructose and inulin. *Gastroenterology* 108:975–82.

Gibson GR, Wang X. 1993. Effects of the *in vitro* fermentation of oligofructose and inulin by bacteria growing in the human large intestine. *J Appl Bacteriol* 75:373–80.

Gibson GR, Wang X. 1994. Regulatory effects of *bifidobacteria* on the growth of other colonic bacteria. *J Appl Bacteriol* 77:412–20.

- Gionchetti P, Rizzello F, Helwig U, Venturi A, Lammers K, Brigidi P, Vitali B, Poggioli G, Miglioli M, Campieri M. 2003. Prophylaxis of pouchitis onset with probiotic therapy: a double-blind, placebo-controlled trial. *Gastroenterology* 124:1202–9.
- Gionchetti P, Rizzello F, Venturi A, Brigidi P, Matteuzzi D, Bazzocchi G, Poggioli G, Miglioli M, Campieri M. 2000. Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double blind, placebo–controlled trial. *Gastroenterology* 119:305–9.
- Goldin BR. 1986. In situ bacterial metabolism and colon mutagens. *Annu Rev Microbiol* 40:367–93.
- Guandalini S, Pensabene L, Zikri MA, Dias JA, Casali LG, Hoekstra H, Kolacek S, Massar K, Micetic-Turk D, Papadopoulou A, de Sousa JS, Sandhu B, Szajewska H, Weizman Z. 2000. *Lactobacillus* GG administered in oral rehydration solution to children with acute diarrhea: a multicenter European trial. *J Pediatr Gastroenterol Nutr* 30:54–60.
- Guarino A, Canani RB, Spagnuolo MI, Albano F, Benedetto L. 1997. Oral bacterial therapy reduces the duration of symptoms and of viral excretion in children with mild diarrhea. *J Pediatr Gastroenterol Nutr* 25:516–9.
- Guarner F, Malagelada JR. 2003. Gut flora in health and disease. *Lancet* 361:512–9.
- Guarner F, Schaafsma GJ. 1998. Probiotics. *Int J Food Microbiol* 39:237–8.
- Guigoz Y, Rochat F, Perruisseau-Carrier G, Rochat I, Schiffrin EJ. 2002. Effects of oligosaccharide on the faecal flora and non-specific immune system in elderly people. *Nutr Res* 22:13–25.

- Hayatsu H, Hayatsu T. 1993. Suppressing effect of *Lactobacillus casei* administration on the urinary mutagenicity arising from ingestion of fried ground beef in the human. *Cancer Lett* 73:173–9.
- Hilton E, Kolakowski P, Singer C, Smith M. 1997. Efficacy of *Lactobacillus* GG as a diarrheal reventive in travelers. *J. Travel Med* 4:41–3.
- Hughes DB, Hoover DG. 1995. Viability and enzymatic activity of *Bifidobacteria* in milk. *J Dairy Sci* 78:268–76.
- Ibrahim SA, Bezkorovainy A. 1993. Survival of *Bifidobacteria* in the presence of bile salt. *J Sci Food Agri* 62:351–4.
- Ishibashi N, Shimamura S. 1993. *Bifidobacteria*: Research and development in Japan. *Food Technol* 47(66):126–34.
- Isolauri E, Arvola T, Sutas Y, Moilanen E, Salminen S. 2000. Probiotics in the management of atopic eczema. *Clin Exp Allergy* 30:1604–10.
- Isolauri E, Juntunen M, Rautanen T, Sillanauke P, Koivula, T. 1991. A human *Lactobacillus* strain (*Lactobacillus casei* sp strain GG) promotes recovery from acute diarrhea in children. *Pediatrics* 88:90–7.
- Janer C, Peláez C, Requena T. 2004. Caseinomacropeptide and whey protein concentrate enhance *Bifidobacterium lactis* growth in milk. *Food Chem* 86:263–7.
- Jiang T, Mustapha AM, Savaiano DA. 1996. Improvement of lactose digestion in humans by ingestion of unfermented milk containing *Bifidobacterium longum*. *J Dairy Sci* 79:750–7.

- Jiang T, Savaiano DA. 1997. Modification of colonic fermentation by *Bifidobacteria* and pH *in vitro*. Impact on lactose metabolism, short-chain fatty acid, and lactate production. Dig Dis Sci 42:2370–7.
- Kalliomäki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E. 2001. Probiotics in primary prevention of atopic disease: a randomized placebo-controlled trial. Lancet 357:1076–9.
- Kießling G, Schneider J, Jahreis G. 2002. Long-term consumption of fermented dairy products over 6 months increases HDL cholesterol. Eur J Clin Nutr 56:843–9.
- Kim H, Kwack K, Kim DY, Ji GE. 2005. Oral probiotic bacterial administration suppressed allergic responses in an ovalbumin-induced allergy mouse model. FEMS Immunol Med Microbiol 45:259–67.
- Kirjavainen PV, Ouwehand AC, Isolauri E, Salminen SJ. 1998. The ability of probiotic bacteria to bind human intestinal mucus. FEMS Microbiol Lett 167:185–9.
- Klaver FAM, Kingma F, Weerkamp AH. 1993. Growth and survival of *bifidobacteria* in milk. Neth Milk Dairy J 47:151–64.
- Kruis W, Schutz E, Fric P, Fixa B, Judmaier G, Stolte M. 1997. Double-blind comparison of an oral *Escherichia coli* preparation and mesalazine in maintaining remission of ulcerative colitis. Aliment Pharmacol Ther 11:853–8.
- Kurmann JA, Rašić JL. 1991. The health potential of products containing *bifidobacteria*. In: Robinson RK, editor. Therapeutic properties of fermented milks. London, England: Elsevier Applied Science Publishers, Ltd. p 117–58.
- Lankaputhra WEV, Shah NP. 1995. Survival of *Lactobacillus acidophilus* and *bifidobacterium* spp. in the presence of acid and bile salts. Cult Dairy Prod J 30:2–7.

- Leahy SC, Higgins DG, Fitzgerald GF, Sinderen D. van. 2005. Getting better with *bifidobacteria*. J Appl Microbiol 98:1303–15.
- Lee KY, Heo TR. 2000. Survival of *Bifidobacterium longum* immobilized in calcium alginate beads in simulated gastric juice and bile salt solution. Appl Env Microbiol 66:869–873.
- Lepercq P, Relano P, Cayuela C, Juste C. 2004. *Bifidobacterium animalis* strain DN-173 010 hydrolyses bile salts in the gastrointestinal tract of pigs. Scand J Gastroenterol 39:1266 – 71.
- Lian WC, Hsiao HC, Chou CC. 2003. Viability of microencapsulated *bifidobacteria* in simulated gastric juice and bile solution. Int J Food Microbiol 86:293–301.
- Lidbeck A, Overvik E, Rafter J, Nord CE, Gustafsson JA. 1992. Effect of *Lactobacillus acidophilus* supplements on mutagen excretion in feces and urine in humans. Microbial Ecol Health Dis 5:59–67.
- Lilly DM, Stillwell RH. 1965. Probiotics: growth promoting factors produced by microorganisms. Science 147:747–8.
- Lin DC. 2003. Probiotics as functional foods. Nutr Clin Pract 18:497–506.
- Lin SY, Ayres JW, Winkler Jr. W, Sandine W. 1989. *Lactobacillus* effects on cholesterol: *In vitro* and *in vivo* results. J Dairy Sci 72:2885–99.
- Losada MA, Olleros T. 2002. Towards a healthier diet for the colon: the influence of fructooligosaccharides and *lactobacilli* on intestinal health. Nutr Res 22:71–84.
- Majamaa H, Isolauri E. 1997. Probiotics: A novel approach in the management of food allergy. J Allergy Clin Immunol 99:179–85.

- Marteau P, Boutron-Ruault MC. 2002. Nutritional advantages of probiotics and prebiotics. *Br J Nutr* 87:153S–7S.
- Marteau P, Flourie B, Pochart P, Chastang C, Desjeux JF, Rambaud JC. 1990. Effect of the microbial lactase (*EC* 3.2.1.23) activity in yoghurt on the intestinal absorption of lactose: an in vivo study in lactase-deficient humans. *Br J Nutr* 64:71–9.
- Marteau P, Pochart P, Bouhnik Y, Rambaud JC. 1993. The fate and effects of transiting, non-pathogenic micro-organisms in the human intestine. In: Simopoulos AP, Corring T, R  rat A, editors. *World Review of Nutrition and Dietetics: Intestinal Flora, Immunity, Nutrition and Health*. Vol. 74. London: Krager. p 1 – 21.
- Marteau P, Seksik P, Jian R. 2002. Probiotics and intestinal health effects: a clinical perspective. *Br J Nutr* 88:S51–7.
- Marteau P, Vesa T, Rambaud JC. 1997. Lactose maldigestion. In: Fuller R, editor. *Probiotics 2: Applications and practical aspects*. London: Chapman & Hall. p 65–88.
- Mart  n-Diana AB, Janer C, Pel  ez C, Requena T. 2003. Development of a fermented goat’s milk containing probiotic bacteria. *Int Dairy J* 13:827–33.
- Matricardi P, Bjorksten B, Bonini S, Bousquet J, Djukanovic R, Dreborg S, Gereda J, Malling HJ, Popov T, Raz E, Renz H. 2003. Microbial products in allergy prevention and therapy. *Allergy* 58:461–71.
- Matsumoto M, Ohishi H, Bento Y. 2004. H⁺-ATPase activity in *Bifidobacterium* with special reference to acid tolerance. *Int J Food Microbiol* 93:109–13.
- McIntosh GH, Royle PJ, Playne MJ. 1999. A probiotic strain of *L. acidophilus* reduces DMH-induced large intestinal tumors in male Sprague–Dawley rats. *Nutr Cancer* 35:153–9.

Meile L, Ludwig W, Rueger U, Gut C, Kaufmann P, Dasen G, Wenger S, Teuber M. 1997.

Bifidobacterium lactis sp. nov., a moderately oxygen tolerant species isolated from fermented milk. Syst Appl Microbiol 20:57–64.

Metchnikoff E. 1907. The prolongation of life. New York: GP Putman's Sons. p 161–183.

Modler HW, McKellar RC, Yaguchi M. 1990. *Bifidobacteria* and bifidogenic factors. Can Inst Food Sci Technol J 23:29–41.

Molis C, Flourié B, Ouarne F, Gailing MF, Lartigue S, Guibert A, Bornet F, Galmiche JP.

1996. Digestion, excretion, and energy value of fructooligosaccharides in healthy humans. Am J Clin Nutr 64:324–8.

O'Mahony L, Feeney M, O'Halloran S, Murphy L, Kiely B, Fitzgibbon J, Lee G, O'Sullivan

G, Shanahan F, Collins JK. 2001. Probiotic impact on microbial flora, inflammation and tumour development in IL–10 knockout mice. Aliment Pharmacol Ther 15:1219–25.

Oberhelman RA, Gilman RH, Sheen P, Taylor DN, Black RE, Cabrera L, Lescano AG, Meza

R, Madico G. 1999. A placebo–controlled trial of *Lactobacillus* GG to prevent diarrhea in undernourished Peruvian children. J Pediatr 134:15–20.

Oksanen PJ, Salminen S, Saxelin M, Hamalainen P, Ihantola-Vormisto A, Muurasniemi-

Isoviita L, Nikkari S, Oksanen T, Porsti I, Salminen E. et al. 1990. Prevention of travellers' diarrhoea by *Lactobacillus* GG. Ann Med 22:53–6.

Ouwehand AC, Salminen S, Isolauri E. 2002. Probiotics: an overview of beneficial effects.

Antonie van Leeuwenhoek 82:279–89.

Parashar UD, Hummelman EG, Bresee JS, Miller MA, Glass RI. 2003. Global illness and

deaths caused by rotavirus disease in children. Emerg Infect Dis 9:565–72.

- Park HK, So JS, Heo TR. 1995. Acid adaptation promotes survival of *Bifidobacterium breve* against environmental stress. *Food Biotechnol* 4:226–30.
- Passerat B, Desmaison AM. 1995. Lactase activity of *Bifidobacterium bifidum*. *Nutr Res* 15:1287–95.
- Pérez PF, Minnaard Y, Disalvo EA, De Antoni GL. 1998. Surface properties of *Bifidobacterial* strains of human origin. *Appl Environ Microbiol* 64:21–6.
- Petschow BW, Talbott D. 1990. Growth promotion of *Bifidobacterium* species by whey and casein fractions from human and bovine milk. *J Clin Microbiol* 28:287–92.
- Plummer S, Weaver MA, Harris JC, Phillipa D, Hunter J. 2004. *Clostridium difficile* pilot study: effects of probiotic supplementation on the incidence of *C. difficile* diarrhea. *Int Microbiol* 7:59–62.
- Pochart P, Marteau P, Bouhnik Y, Goderel I, Bourlioux P, Rambaud JC. 1992. Survival of *Bifidobacteria* ingested via fermented milk during their passage through the human small intestine: an *in vivo* study using intestinal perfusion. *Am J Clin Nutr* 55:78–80.
- Present DH. 1998. Ciprofloxacin as a treatment for ulcerative colitis—not yet. *Gastroenterology* 115:1289–91.
- Rambaud JC, Bouhnik Y, Marteau P, Pochart P. 1993. Manipulation of the human gut microflora. *Proc Nutr Soc* 52:357–66.
- Rao AV, Shiwnarain N, Maharaj I. 1989. Survival of microencapsulated *Bifidobacterium pseudolongum* in simulated gastric and intestinal juices. *Can Inst Food Sci Technol J* 22: 345–9.

- Rašić JL, Kurmann JA. 1983. *Bifidobacteria* and their role: Microbiological, nutritional-physiological, medical and technological aspects and bibliography. Basel, Switzerland: Birkhauser Verlag. p 1–21.
- Rašić JL, Vujicic IF, Skrinjar M, Vulic M. 1992. Assimilation of cholesterol by some cultures of lactic acid bacteria and *Bifidobacteria*. *Biotechnol Lett* 14:39–44.
- Raza S, Graham SM, Allen SJ, Sultana S, Cuevas L, Hart CA. 1995. *Lactobacillus* GG promotes recovery from acute nonbloody diarrhea in Pakistan. *Pediatr Infect Dis J* 14:107–11.
- Reddy BS, Rivenson A. 1993. Inhibitory effect of *Bifidobacterium longum* on colon, mammary and liver carcinogenesis induced by 2-amino-3-methylimidazo[4,5-f]quinoline, a food mutagen. *Cancer Res* 53:3914 –8.
- Rembacken B, Snelling A, Hawkey P, Chalmers D, Axon A. 1999. Non-pathogenic *Escherichia coli* versus mesalazine for the treatment of ulcerative colitis: a randomised trial. *Lancet* 354:635–9.
- Reuter G. 1971. Designation of type strains for *Bifidobacterium* species. *Int J System Bacteriol* 21:273–5.
- Roberfroid MB. 2000a. Chicory fructooligosaccharides and the gastrointestinal tract. *Nutrition*. 16:677–9.
- Roberfroid MB. 2000b. Prebiotics and probiotics: are they functional foods? *Am J Clin Nutr* 71:1682S–7S.
- Rogosa M. 1974. Genus III, *Bifidobacterium* Orla-Jensen. In: Buchanan RE, Gibbons NE, editors. *Bergey's Manual of determinative bacteriology*. 8th ed. Baltimore: Williams & Wilkins. p 669-76.

- Rolfe RD. 2000. The role of probiotic cultures in the control of gastrointestinal health. *Am Soc Nutr Sci* 130:396S–402S.
- Ross RP, Desmond C, Fitzgerald GF, Stanton C. 2005. Overcoming the technological hurdles in the development of probiotic foods. *J Appl Microbiol* 98:1410–7.
- Rossouw JE, Burger EM, Van der Vyver P, Ferreira J. 1981. The effect of skim milk, yoghurt, and full cream milk on human serum lipids. *Am J Clin Nutr* 34:351–6.
- Saarela M, Mogensen G, Fondén R, Mättö J, Mattila-Sandholm T. 2000. Probiotic bacteria: safety, functional and technological properties. *J Biotechnol* 84:197–215.
- Saavedra JM, Bauman NA, Oung I, Perman JA, Yolken RH. 1994. Feeding of *Bifidobacterium bifidum* and *Streptococcus thermophilus* to infants in hospital for prevention of diarrhoea and shedding of rotavirus. *Lancet* 344:1046–9.
- Saavedra JM. 2001. Clinical applications of probiotic agents. *Am J Clin Nutr* 73(S):1147–51.
- Salminen S, Ouwehand AC, Isolauri E. 1998. Clinical applications of probiotic bacteria. *Int Dairy J* 8:563–72.
- Sanders ME, Walker DC, Walker KM, Aoyama K, Klaenhammer TR. 1996. Performance of commercial cultures in fluid milk applications. *J Dairy Sci* 79:943–55.
- Scardovi V. 1984. Genus *Bifidobacterium* Orla-Jensen. In: Kreig NR, Holt JG, editors. *Bergey's manual of systematic bacteriology*, Vol. 1. Baltimore, MD: Williams & Wilkins. p 1418–34.
- Scheinbach S. 1998. Probiotics: functionality and commercial status. *Biotechnol Adv* 16:581–608.
- Schrezenmeir J, de Vrese M. 2001. Probiotics, prebiotics, and synbiotics—approaching a definition. *Am J Clin Nutr* 73:361S–4S.

- Servin AL. 2004. Antagonistic activities of *lactobacilli* and *bifidobacteria* against microbial pathogens. FEMS Microbiol Rev 28:405–40.
- Shanahan F. 2002. Probiotics and inflammatory bowel diseases: from fads and fantasy to facts and future. Br J Nutr 88:S5–S9.
- Shanahan F. 2004. Probiotics in inflammatory bowel disease-therapeutic rationale and role. Adv Drug Deliv Rev 56:809–18.
- Shimamura S, Abe F, Ishibashi N, Miyakawa H, Yaeshima, TA, Tomita M. 1992. Relationship between oxygen sensitivity and oxygen metabolism of *Bifidobacterium* species. J Dairy Sci 75:3296–306.
- Shin HS, Lee JH, Pestka JJ, Ustunol Z. 2000a. Growth and viability of commercial *Bifidobacterium* spp in skim milk containing oligosaccharides and inulin. J Food Sci 65:884–7.
- Shin HS, Lee JH, Pestka JJ, Ustunol Z. 2000b. Viability of *Bifidobacteria* in commercial dairy products during refrigerated storage. J Food Prot 63:327–31.
- Shornikova AV, Casas IA, Mykkanen H, Salo E, Vesikari T. 1997. Bacteriotherapy with *Lactobacillus reuteri* in rotavirus gastroenteritis. Pediatr Infect Dis J 16:1103–7.
- Siitonen S, Vapaatalo H, Salminen S, Gordin A, Saxelin M, Wikberg R, Kirkkola AL. 1990. Effect of *Lactobacillus* GG yoghurt in prevention of antibiotic associated diarrhoea. Ann Med 22:57–9.
- Silva AM, Bambirra EA, Oliveira AL, Souza PP, Gomes DA, Vieira EC, Nicoli JR. 1999. Protective effect of bifidus milk on the experimental infection with *Salmonella enteritidis* subsp. *Typhimurium* in conventional and gnotobiotic mice. J Appl Microbiol 86:331–6.

- Silva AM, Barbosa FHF, Duarte R, Vieira LQ, Arantes RME, Nicoli JR. 2004. Effect of *Bifidobacterium longum* ingestion on experimental *Salmonellosis* in mice. *J Appl Microbiol* 97:29–37.
- Simpson, PJ, Ross RP, Fitzgerald GF, Stanton C. 2004. *Bifidobacterium psychraerophilum* sp. nov and *Aeriscardovia aeriphila* gen. nov., sp. nov., isolated from a porcine caecum. *Int J Syst Evol Microbiol* 54:401–6.
- Singh J, Rivenson A, Tomita M, Shimamura S, Ishibashi N, Reddy BS. 1997. *Bifidobacterium longum*, a lactic acid-producing intestinal bacterium inhibits colon cancer and modulated the intermediate biomarkers of colon carcinogenesis. *Carcinogenesis* 18:833–41.
- Strachan DP. 1989. Hay fever, hygiene, and household size. *Br Med J* 299:1259–60.
- Sullivan Å, Nord CE. 2005. Probiotics and gastrointestinal diseases. *J Intern Med* 257:78–92.
- Sun W, Griffiths MW. 2000. Survival of *bifidobacteria* in yogurt and simulated gastric juice following immobilization in gellan–xanthan beads. *Int J Food Microbiol* 61:17–25.
- Surawicz CM. 2003. Probiotics, antibiotic-associated diarrhoea and *Clostridium difficile* diarrhoea in humans. *Best Prac Res Clin Gastroenterol* 17:775–83.
- Svensson U. 1999. Industrial Perspective. In: Tannock GW, editor. *Probiotics: A critical review*. Portland, OR: Horizon Scientific Press. p 57–64.
- Talwalkar A, Kailasapathy K. 2003. Metabolic and biochemical responses of probiotic to oxygen. *J Dairy Sci* 86:2537–46.
- Tannock GW. 2002. The *Bifidobacterial* and *Lactobacillus* microflora of humans. *Clin Rev Allergy Immunol* 22:231–52.

- Tavan E, Cayuela C, Antoine JM, Trugnan G, Chaugier C, Cassand P. 2002. Effects of dairy products on heterocyclic aromatic amine-induced rat colon carcinogenesis. *Carcinogenesis* 23:477–83.
- Thom T, Haase N, Rosamond W, Howard VJ, Rumsfeld J, Manolio T, Zheng ZJ, Flegal K, O'Donnell C, Kittner S, Lloyd-Jones D, Goff Jr DC, Hong Y. 2006. Heart disease and stroke statistics—2006 update. A report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 113:85–151.
- Thompson LU, Jenkinds DJ, Amer MA, Reicher R, Jenkins A, Kamulsky J. 1982. The effect of fermented and unfermented milks on serum cholesterol. *Am J Clin Nutr* 36:1106–11.
- Touré R, Kheadr E, Lacroix C, Moroni O, Fliss I. 2003. Production of antibacterial substances by *bifidobacterial* isolates from infant stool active against *Listeria monocytogenes*. *J Appl Microbiol* 95:1058–69.
- Tuomola EM, Salminen SJ. 1998. Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. *Int J Food Microbiol* 41:45–51.
- Vesa TH, Marteau P, Korpela R. 2000. Lactose intolerance. *J Am Coll Nutr* 19:165S–75S.
- Vinderola CG, Costa GA, Regenhardt S, Reinheimer JA. 2002. Influence of compounds associated with fermented dairy products on the growth of lactic acid starter and probiotic bacteria. *Int Dairy J* 12: 579–89.
- [WHO] World Health Organization. 1995. The treatment of diarrhoea - A manual for physicians and other senior health workers WHO/CDR/95.3. Available from: <http://www.who.int/en/>. Accessed Jan 31, 2006.

- Xiao JZ, Kondo S, Takahashi N, Miyaji K, Oshida K, Hiramatsu A, Iwatsuki K, Kokubo S, Hosono A. 2003. Effects of milk products fermented by *Bifidobacterium longum* on blood lipids in rats and healthy adult male volunteers. J Dairy Sci 86:2452–61.
- Young RJ, Whitney DB, Hanner TL, Antonson DL, Lupo JV, Vanderhoof JA. 1998. Prevention of antibiotic-associated diarrhea utilizing *Lactobacillus* GG. Gastroenterology 114:A435.

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